Final Report

Phytoremediation for the Containment and Treatment of Energetic and Propellant Material Releases on Testing and Training Ranges

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The overall objective of the proposed research is to understand the mechanisms by which toxic energetic compounds, known to be susceptible to biodegradation, are actually detoxified in contaminated subsurface soils at DoD firing ranges by plants native to the site, either by direct uptake and transformation in plant tissues, or by microbial activity in the rhizosphere. The specific objectives of the research are to determine: (1) whether plants significantly improve biodegradation of explosives using actual soils and plants from representative sites; (2) the respective contribution of plants and soil microbes in the process; and (3) whether the aging of explosives affects the biodegradation process. Additionally, a field-scale implementation of phytoremediation was performed. The specific objectives of this field study are to: (1) determine if the implementation of phytoremediation study significantly improves the biodegradation of explosives in soil through a field study; (2) determine whether plants can significantly uptake and degrade explosives in the field; and (3) compare fate and transport processes in laboratory studies using actual soils from the site of the field study with the field demonstration results.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

2-ADNT: 2-amino-4,6-dinitrotoluene

2-NT: 2-nitrotoluene

3-NT: 3-nitrotoluene

4-ADNT: 4-amino-2,6-dintrotoluene

4-NT: 4-nitrotoluene

2,4-DNT: 2,4-dinitrotoluene

2,6-DNT: 2,6-dinitroltoluene

AcCN: Acetonitrile

AFB: Air Force Base

DANT: diaminonitrotoluene

DI: Deionized water

DM: Dorovan muck

DNB: 1,3-dinitrobenzene

HMX: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine

HPLC: High performance liquid chromatography

LC/MS: Liquid chromatography/mass spectrometry

LS: Lakeland soil

LSC: Liquid scintillation counter

NB: nitrobenzene

TNB: 1,3,5-trinitrobenzene

TNT: 2,4,6-trinitroltoluene

RDX: hexahydro-1,3,5-trinitro-1,3,5-triazine

KEYWORDS

TNT; 2,4,6-trinitrotoluene; RDX; hexahydro-1,3,5-trinitro-1,3,5-triazine; HMX; octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; Eglin Air Force Base; field study; phytoremediation; hybrid poplar; bahiagrass; switchgrass; phosphor imager autoradiography; autoradiography; biodegradation; T-RFLP; phytotoxicity; microarray.

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I. Abstract

The overall objective of the proposed research is to understand the mechanisms by which toxic energetic compounds, known to be susceptible to biodegradation, are actually detoxified in contaminated subsurface soils at DoD firing ranges by plants native to the site, either by direct uptake and transformation in plant tissues, or by microbial activity in the rhizosphere. The specific objectives of the research are to determine: (1) whether plants significantly improve biodegradation of explosives using actual soils and plants from representative sites; (2) the respective contribution of plants and soil microbes in the process; and (3) whether the aging of explosives affects the biodegradation process. Additionally, a field-scale implementation of phytoremediation was performed. The specific objectives of this field study are to: (1) determine if the implementation of phytoremediation study significantly improves the biodegradation of explosives in soil through a field study; (2) determine whether plants can significantly uptake and degrade explosives in the field; and (3) compare fate and transport processes in laboratory studies using actual soils from the site of the field study with the field demonstration results.

Biodegradation experiments were conducted at the laboratory, greenhouse, and field scale. The laboratory and greenhouse studies utilized soil materials and plant species from Eglin Air Force Base (Eglin AFB), the site of the field study. Several biodegradation studies were performed, including the exposure of unplanted soils to TNT, RDX, and HMX and the exposure of planted soils to TNT and RDX. Weathering and sequestration of chemicals were simulated by aging artificially contaminated soil in the laboratory prior to the start of degradation experiments. Hybrid poplar and switchgrass was exposed to radiolabeled TNT and RDX and were then explored using phosphor imager autoradiography in order to determine pathway and transport mechanisms. The microbial communities of native Eglin AFB soils were “finger printed” using T-RFLP. The properties of each soil type were analyzed and the bioavailability of TNT and RDX was determined by performing sorption studies on these soils. Gene expression in hybrid poplars exposed to TNT was also investigated. A field study consisting of three 0.4 acre plots were planted with Bahiagrass (*Paspalum notatum*) Pensacola with biannual sampling over 18 months. Both soil and plant samples were taken from each plot, as well as unplanted perimeter soil samples as a control.

The biodegradation studies found that TNT was readily transformed and degraded by microbial communities in native Eglin AFB soils, while RDX and HMX remained recalcitrant under unplanted conditions. Additional biodegradation studies found that the RDX concentration in soil rapidly decreased in the presence of Bahiagrass Pensacola and hybrid poplar. Phosphor imager autoradiography showed the majority of TNT remains in or bonded to the root tissue of hybrid poplar and switchgrass, while RDX is readily transported to the leaf or blade. Microautoradiographs showed the translocation of RDX or its metabolites into chloroplasts or were incorporated into plant structure. T-RFLP analysis of soils found little diversity in Lakeland soil compared to the much greater diversity found in Dorovan muck. This was expected given the higher organic and nutrient levels in Dorovan muck. In the gene expression study, several new genes were demonstrated to be implicated in the detoxification and metabolism of TNT by hybrid poplar. The results of the field study showed that TNT was transformed in the soil with no apparent treatment benefit in the planted areas and both RDX and HMX migrated downward through the soil before Bahiagrass Pensacola could effectively treat

the compounds. There was some evidence that the application of high carbon content soil, in which the Bahiagrass was established, slowed the migration of TNT and RDX.

Phytoremediation is a cost-effective and sustainable long-term strategy for management of risk at testing and training ranges. While phytoremediation was not effective in treating explosives contamination in the sandy soil at Eglin AFB, it is believed that the treatment may be effective in different soils or using different plant species. The work at Eglin AFB investigated phytoremediation holistically and representatively. This research attempts to account for all of the many factors influencing phytoremediation at Eglin AFB and use factors that are specific to the site. The tools and methods developed in this research lay groundwork for creating standards in phytoremediation research. Sites of interest for phytoremediation should be fully characterized from native soils to native plant species in order to devise a fully integrated approach and predict the success of the treatment technology. This project provides new insights into the mechanisms underlying phytoremediation of explosives and propellants in the field.

II. Objectives

The overall objective of the proposed research is to understand the mechanisms by which toxic energetic compounds, known to be susceptible to biodegradation, are actually detoxified in contaminated subsurface soils at DoD firing ranges by plants native to the site, either by direct uptake and transformation in plant tissues, or by microbial activity in the rhizosphere. The specific objectives of the research are to determine: (1) whether plants significantly improve biodegradation of explosives using actual soils and plants from representative sites; (2) the respective contribution of plants and soil microbes in the process; and (3) whether the aging of explosives affects the biodegradation process. Additionally, a field-scale implementation of phytoremediation was performed. The specific objectives of this field study are to: (1) determine if the implementation of phytoremediation study significantly improves the biodegradation of explosives in soil; (2) determine whether plants can significantly uptake and degrade explosives in the field; and (3) compare fate and transport processes in laboratory studies using actual soils from the site of the field study with the field demonstration results.

III. Background

Toxic explosives and propellants, such as TNT, RDX, and HMX, contaminate military training ranges worldwide and are known to be biodegraded by plants and microbes in the laboratory. Paradoxically, they are also notoriously persistent under actual field conditions at firing ranges. They can migrate from the source and pose a hazard to humans and ecosystems. The central hypothesis of this project is that phytoremediation can be used to significantly improve the *in situ* biodegradation and containment of explosives in contaminated soils at testing and training ranges. Phytoremediation is a cost-effective and sustainable long-term strategy for management of risk at ranges.

This project seeks to move phytoremediation closer to actual use as a long-term, sustainable strategy for testing and training ranges. The project, ER-1499, is a logical extension of CUSON-1317, "Identification of Metabolic Routes and Catabolic Enzymes Involved in the Phytoremediation of Nitro-Substituted Explosives TNT, RDX, and HMX", which showed that plants can uptake and metabolize these chemicals under controlled laboratory conditions using model plants and tissue cultures. The current project focuses on two important elements of the phytoremediation process: the soil and rhizosphere microbial community. Laboratory experiments using real soil materials and plant species native to an explosives contaminated site will add to the applicability of our findings. Finally, the implementation of phytoremediation in a field-scale experiment will allow for the application of the knowledge learned from the biodegradation studies in order to better define the processes occurring in the natural system.

Eglin Air Force Base (AFB), the location of the field study, is on the panhandle of Florida adjacent to the towns of Niceville and Valparaiso as shown in Figure 1. The base occupies 724 square miles of land as well as nearly 98,000 square miles of air space over the Gulf of Mexico making it one of the largest military installations in the world. The base also houses the headquarters of the Air Armament Center, responsible for the development, acquisition, testing, and fielding of all air-delivered non-nuclear weapons for the United States and allies. In addition, most of the undeveloped land on the base is home to endangered plants and animals such as the long leaf pine, red-cockaded woodpecker, bald eagle, piping plover, Okaloosa darter, Gulf sturgeon, flatwoods salamander, Eastern indigo snake, loggerhead sea turtle, green sea turtle, leatherback sea turtle, and the Florida perforate lichen (Jacobson & Marynowski, 2002). Many of these areas are open to the public for recreational uses such as hunting, fishing, camping, hiking, and wildlife observation.

Figure 1. Location of field study within Elgin Air Force Base which is adjacent to Niceville, FL. Map composed on Google Earth®.

In order to protect natural resources and ecosystems at Eglin AFB, a strategy must be developed for the containment and/or treatment of explosive contaminants on testing and training ranges. The firing of munitions, the detonation of ordnance, and the disposal of unexploded ordnance (UXO) result in contamination of soil with explosive compounds (Jenkins et al., 1999; Thiboutot et al., 1998). The energetic compounds most commonly found to contaminate soil on military testing and training ranges include 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). These compounds are persistent environmental contaminants and pose risks to the health of humans and ecosystems (Flokstra, Van Aken, & Schnoor, 2008; Jenkins, Bartolini, & Ranney, 2003). However, plants and microbes have been shown to degrade these explosive compounds (Hawari, Beaudet, Halasz, Thiboutot, & Ampleman, 2000; Hawari et al., 2001; Jenkins et al., 2006; Van Aken & Agathos, 2001).

Phytoremediation is the direct use of living plants for *in situ* (in place) remediation of contaminated soil, sludges, sediments, and groundwater through contaminant removal, degradation, or containment (USEPA, 1999). Due to its ability to continuously treat large areas at low cost with low impact to the site, phytoremediation will be implemented through a field study at Eglin AFB in order to increase the sustainability of range operations.

Properties of TNT, RDX, and HMX

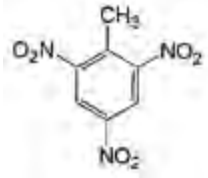
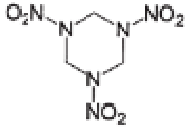
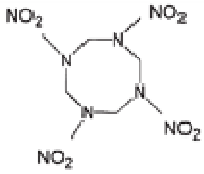
As shown in Table 1, TNT is a nitroaromatic compound, which biodegrades readily under both aerobic and anaerobic soil conditions (Hawari et al., 2000). The mineralization of

TNT is rare because its nitroaromatic ring is resistant to attack (Spain, 1995). However, the nitro groups are easily reducible, resulting in metabolites that are sometimes rather persistent. TNT has also been shown to be toxic. TNT has been shown to cause neurological disorders in workers in large-scale manufacturing operations and can be highly toxic and mutagenic to aquatic species (McCormick, Feeherry, & Levinson, 1976; Won, Disalvo, & Ng, 1976). Table 1 shows the physical and chemical constants of TNT. Losses through volatilization from soil or groundwater to the atmosphere are negligible due to TNT's low vapor pressure and moderately low Henry's law constant. TNT is moderately water soluble and has low partition coefficients which would favor the movement of the compound with little absorption to the soil. However, the products of aerobic and anaerobic biotransformation can irreversibly bind to organic material in the soil (Hawari et al., 2000). TNT is subject to photolysis in an aqueous state (Talmage et al., 1999).

RDX is a cyclic nitramine as shown in Table 1. It is a major component of most military explosives. RDX represents 90% of Composition 4 (C4) and 60% of Composition B (Comp B) (Hewitt et al., 2007). Also from Table 1, it can be seen that RDX does not readily volatilize because of the low vapor pressure and Henry's constant. The solubility and low partition coefficients would suggest RDX exhibits a high degree of mobility in the environment.

HMX is also a cyclic nitramine commonly found in military explosives. The production of HMX also produces small quantities of RDX as a production contaminant. Therefore, both compounds are commonly found together in the environment (Thiboutot, 1998). Octol consists of 70% HMX and 30% TNT (Ampleman, Marois, & Thiboutot, 1999) and is used in rockets, causing extensive contamination on anti-tank ranges (Jenkins et al., 1999). As seen in Table 1, HMX has relatively low water solubility at 6.6 mg/L, but once solubilized the partition coefficients suggest that it will be readily transported through the subsurface.

Table 1. Physical and Chemical Constants for TNT, RDX, and HMX

	TNT	RDX	HMX
Vapor Pressure (mm Hg)	1.99×10^{-4}	4.0×10^{-9}	3.3×10^{-14}
Henry's Law Constants ($atm\text{-}m^3\text{-}mol^{-1}$)	4.57×10^{-7}	1.2×10^{-5}	2.6×10^{-15}
Solubility in H_2O (mg/L, 20 °C)	130	38	6.6
Partition Coefficients			
Log K_{ow}	1.84	0.86	0.13, 0.06
Log K_p	4 - 53	0.83 – 4.13	< 8.7
Log K_{oc}	3.2	0.8 – 4.2	2.8
Structure (Hannink et al., 2002)			

Source: Groom et al. 2002

Remediation Technologies for Explosives

Treatment technologies for the remediation of explosive compounds include biodegradation, bioaugmentation, permeable reactive barriers, pump and treat, soil slurry reactor, excavation, landfilling, incineration, composting, adsorption to activated carbon, and advanced photooxidation processes (Van Aken et al. 2004). Many of these techniques involve invasive work requiring excavation. Since most explosive contamination occurs over a large area, any excavation work will be extremely expensive and ecologically damaging. There are also hazards associated with excavation due to the risk of striking underlying UXO on military testing and training ranges. Phytoremediation usually involves the *in situ* treatment of contaminants meaning it will be comparatively less disruptive to the environment and can be implanted at lower cost (Hannink, Rosser, & Bruce, 2002).

Phytoremediation of Explosives

Phytoremediation is a general term for many processes which plants utilize to transport, transform, or store environmental pollutants. The specific processes include phytoextraction, rhizofiltration, phytostabilization, phytodegradation, rhizodegradation, and phytovolatilization. Phytoextraction refers to the uptake and translocation of contaminants by plant roots. Rhizofiltration is the adsorption or precipitation of contaminants onto the plant roots or absorption into the roots. Phytostabilization is the use of plants to immobilize contaminants by adsorption, absorption, or precipitation of contaminants in the root zone. Phytodegradation is the breakdown of contaminants through metabolic processes within the plant or through interaction with plant exudates in the soil. Rhizodegradation is the breakdown of contaminants in the soil through microbial activity that is enhanced by the presence of the plants. Phytovolatilization is the uptake and transpiration of a contaminant into the atmosphere (USEPA, 1999).

<u>Phytoremediation of TNT</u>

TNT biodegrades readily under both aerobic and anaerobic soil conditions (Hawari et al., 2000). TNT has also been shown to degrade in plant tissues, but very few plants can translocate the TNT to leaves (Schneider, Oltmanns, Radenberg, Schneider, & Mundegar, 1996). As shown by phosphor imager autoradiography, TNT remains in roots with little to no translocation to leaves and stems (Brentner, Mukherji, Walsh, & Schnoor, 2009). This is due to TNT's high biochemical reactivity of the aromatic nitro group which forms oxidative couplings on roots (Thompson, Ramer, & Schnoor, 1998). Common metabolites of TNT include 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT). 2-ADNT and 4-ADNT are formed by aerobic reduction of TNT (Hannink, et al., 2002). 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoleune (2,6-DNT) have also been shown as TNT metabolites in plants. 2,4-DNT and 2,6-DNT have been shown to be potent animal carcinogens (Rickert, Butterworth, Popp, 1984).

<u>Phytoremediation of RDX</u>

RDX is fairly soluble and does not bond well to organic or soil fractions, therefore it is readily translocated by plants. Phosphor imager autoradiography showed that RDX is translocated and stored or transformed in the plant leaves (Brentner et al., 2009). Several studies have shown different degradation pathways once translocated to the leaves. The transformation of RDX to polar metabolites and bound residues containing RDX metabolites has been observed (Hannink et al., 2002; Just & Schnoor, 2004). A pathway resulting in the mineralization of RDX has also been observed (Van Aken, et al., 2004).

Phytoremediation of HMX

HMX exhibits poor solubility. However, when HMX does solubilize it is readily taken up by plants due to its low affinity to bond to organic and sediment particles. This results in the translocation of HMX into the leaves. No degradation or transformation is known to occur before, during, or after translocation. Over half of the HMX contained in leaf tissues was observed to leach out of fallen leaves (Yoon, Oh, Just, & Schnoor, 2002). This is of serious concern if phytoremediation is to be applied for the treatment of HMX.

Phytoremediation of Explosives in Field Studies

There were no examples of field-scale *in situ* phytoremediation to treat explosives contaminated soils in published literature. There have been field studies demonstrating the use of phytoremediation in wetland systems to treat explosives. Phytoremediation was implemented in a wetland system for the treatment of explosives at the Iowa Army Ammunition Plant in Middletown, Iowa producing positive results (McCutcheon & Schnoor, 2003). Following construction of two treatment wetlands, monitoring results conducted for 2 years found no TNT and RDX concentrations above the EPA human health advisory level of 0.002 mg/L when the wetlands were discharging to adjacent surface waters.

IV. Materials and Methods

Bulk Soil Collection

Soil samples were collected during the August 21-22, 2006 field site visit at Eglin AFB. 136.2 kg of Lakeland Sandy Soil was collected at GPS coordinates -86:38:41.053587, 30:35:31.284834 and 63.56 kg of Dorovan Muck Soil was collected at GPS coordinates -86:39:41.424052, 30:35:26.098330. The locations of the collection sites are shown in Figure 4. Soil samples were shipped in 28-quart coolers (4 coolers per soil type) at 4-8 °C from Niceville, FL to The University of Iowa Department of Civil and Environmental Engineering in Iowa City, IA. The soils were stored at 4 °C. A second trip to Eglin AFB to collect more soil samples was completed March 1, 2007. Lakeland soil was collected in the same location as on the previous visit. The previous collection site for Dorovan muck was underneath 2 feet of standing water and could not be resampled. A secondary site, Anderson Pond (GPS coordinates: -86:30:55.614, 30:33:34.746), was used for collection of Dorovan muck. The soil is underwent physical-chemical characterization to confirm that the soil is the same.

Phytotoxicity

Hydroponically grown grasses were initially spiked with concentrations of 0, 2, 5, 10 and 25 mg/L TNT in 0.5x Hoagland hydroponic solution and were placed in a controlled environmental growth chamber at 50% RH, 28°C with a 16:8h light:dark photoperiod (150 μmol s^{-1} m^{-2}). Samples of hydroponic growth medium were taken and filtered (0.22 μm) and analyzed for TNT on HPLC. Grasses were monitored for change in biomass, transpiration, and observable toxicity markers such as chlorosis or mortality.

Biodegradation of TNT by Grass Mixture and Hybrid Poplar

Seeds of *Panicum vigratum* (switchgrass) Alamo and *Paspalum notatum* (bahiagrass) Pensacola were purchased from Adams-Briscoe Seed Company, Jackson, GA. Seeds were spread on perlite soaked in 0.5x Hoagland solution and allowed to sprout in dark conditions for up to 96 hours. Sprouted grasses were grown in perlite under 16:8 photoperiod (150 μmol s^{-1} m^{-2}) for several weeks, alternating watering between 0.5x Hoagland solution and deionized H_2O approximately every 72 hours. Poplar cuttings (*Populus trichocarpa*) were obtained from Segal Ranch Hybrid Poplars nursery (Grandview, WA). Hydroponic poplar plantlets were produced by growing 8-inch dormant cuttings for two weeks in 0.5x modified Hoagland solution under a 16:8 hour light:dark photoperiod.

Soils were homogenized by sieving <2.0 mm and DI-H_2O was added to 70% water content. Each soil type was contaminated separately with radiolabeled [U-^{14}C]-TNT and [U-^{14}C]-RDX at respective concentrations of 100 and 50 mg/kg of soil. Radiolabeled explosive was diluted with non-labeled explosive (20 μL 0.250 μCi/L in 2 mL 50 mg/L TNT or 25 mg/L RDX) dissolved in acetonitrile. The explosives mixture was first mixed vigorously into 6% of soil mass, subsequently blended with the remaining soil for 20 seconds, mixed with a spatula, and then blended again for 20 seconds to obtain a homogenous contamination. Soils were stored in a cooler at 4°C until used in degradation experiments. The final activity of the contaminants in the soil was 11,100 DPM/1g soil. The final volume of contaminated soil was 28L for each soil type and explosive, or 40.6 kg of Lakeland soil and 26.3 kg of Dorovan muck.

Extraction of explosives residues and transformation products was performed using a modified protocol based on Jenkins and Walsh (1987). Samples were composited from eight locations in the pot to account for heterogeneity. Soil samples were dried at room temperature

until a constant weight was achieved. Samples were then ground with a mortar and pestle and extracted with 10 mL of acetonitrile to 1 g soil in a refrigerated ultrasonic bath overnight. The samples were filtered under 0.2 μm. The filtrate was analyzed by HPLC. Filters with solid, non-extractable radioactive residues were mineralized using a biological oxidizer and analyzed by a liquid scintillation counter.

TNT, and its metabolites ADNT and DANT, were analyzed by HPLC (HP Series 1100; Hewlett Packard, Palo Alto, CA) using a C_{18} Supelcosil® LC-18 column (Supelco, Bellfonte, PA). The system was equipped with a UV-visible photodiode array detector (HP Series 1100). The mobile phase consisted of acetonitrile:distilled water 50:50 v/v running at a flow rate of 1.0 mL min^{-1}.

Samples of Hoagland solution were mixed with one volume of acetonitrile and filtered on 0.2 μm. Root tissues were ground under liquid nitrogen, mixed with 1 volume of acetonitrile (v/w) and glass beads, then homogenized in a bead beater. Extracts were then sonicated overnight, centrifuged (13,000 rpm), and filtered (0.2 μm).

Total radioactivity in extracts was analyzed with a Beckman liquid scintillation counter (LSC) LS6000IC (Beckman Coulter, Fullerton, CA) using Ultima Gold XR scintillation cocktail (Packard Bioscience). Radioactivity association with exposed plant tissues was analyzed by combustion using a biological oxidizer (Harvey OX6000; RJ Harvey Instrument, Hillsdale, NJ) with resulting $^{14}CO_2$ trapped in 10 mL of scintillation cocktail.

Phosphor Imager Autoradiography and Microscopy

Autoradiography has been used to investigate the fate of toxic xenobiotic compounds in plants (Woodard-Blankenship, 1995; Best et al., 1999; Feng, 2004; Hornik, 2005; Nepovim, 2005; Adamia et al.; 2006; Chrikishvili, 2006; Cosio, 2006; Maracci, 2006; Soudek, 2006). These studies utilized x-ray, traditional, and emulsion film technologies that require lengthy exposure periods (20-60 days). The development of phosphor imager autoradiography further opens the possibilities of utilizing this technique for localization of contaminants in plants. The most popular storage plate is composed of europium (Eu^{2+}) activated barium fluorobromide (BaFBr) phosphor which absorbs radiation emitted from a sample. The stored radiation energy is released as photons when the phosphor is scanned with visible light. The excitation wavelength of Eu:BaFBr photon is 633 nm and its emission wavelength is 390 nm. The energy of a photon is recorded and converted to a visible image (Schweizer, 2001). Major advantages of using phosphor storage plates for ^{14}C autoradiography are greater sensitivity (100 to 1000 times more sensitive than the film and fast analysis), requires one tenth of the time compared to traditional film (Kanekal, 1995; Cole, 2003). Only a handful of studies have begun to use phosphor imager technology to monitor uptake and transport of xenobiotics in plants (Sulmon, 2007; Vila, 2007).

Poplar cuttings (*Populus deltoides x nigra,* DN34) were obtained from Segal Ranch Hybrid Poplars nursery (Grandview, WA). Hydroponic poplar plantlets were produced by growing 20 cm dormant cuttings for four weeks in 0.5x Hoagland solution. Switchgrass (*Paspalum notatum*) Alamo was grown from seeds supplied by Adams-Briscoe Seed Co. (Jackson, GA). Switchgrass seeds were sprouted on perlite soaked in 0.25x modified Hoagland solution before being transferred to Erlenmeyer flasks of 0.5x Hoagland solution. All plants were maintained under a 16:8 hour light:dark photoperiod (150 μmol s^{-1} m^{-2}).

RDX was synthesized according to Amplemann et al. (1995) and purified by recrystallization. Synthesized RDX was 99% pure or higher as indicated by HPLC analysis.

TNT was purchased from Fischer Scientific, prepared by SpexCertiPrep. Radioactive [U-^{14}C]-RDX and [U-^{14}C]-TNT, each 0.25 mCi mmol^{-1}, were purchased from PerkinElmer (Boston, MA).

Plants were exposed to a mixture of cold and [U-^{14}C]-labeled TNT or RDX by spiking hydroponic growth medium with explosive solutions dissolved in acetonitrile. A control plant which had been exposed to only cold RDX or TNT was prepared for background analysis. After the exposure period, poplar leaf, stem, and roots were excised at the base from the woody stem or whole switchgrass plants, and then dried at 50°C for 8 hours under vacuum. Dried plants were exposed to a phosphor screen overnight. The screen was scanned using a phosphor imager (Typhoon 9410, GE Healthcare) with 50 μm resolution. Control plants exposed to non-radiolabeled RDX or TNT yielded a blank autoradiogram. For semi-quantitative analysis, aliquots of known activity were deposited onto Whatman 3MM paper and treated under the same conditions as the plants.

After the exposure period, plant leaf was excised, fixed, embedded in paraffin, and sectioned as described by Tanaka et al. (2008). Prior to use, the slides were de-waxed, hydrated, and dried. In a dark room, the slides were dipped into liquid NTB2 emulsion film (Kodak, Rochester, NY), dried for 2 hours and placed in a light-tight box (Tanaka et al., 2008). The box was kept at 4˚C for two weeks. D-19 developer (Kodak) and quick fixer (Kodak) was used to develop images and post staining was performed with Fast green FCF (0.02%) (Tanaka et al., 2008).

A leaf was sacrificed after 24, 48 and 96 hours of exposure and the rest was collected after 120 hours of exposure after in-tact autoradiography images were taken. The stem was divided into three fractions: lower, middle, and upper. Leaves and stem fractions were dried at 35 °C to a constant weight (Thompson et al., 1999). Each sample was carefully weighed to no more than 0.05 g. The sample was combusted using a biological oxidizer Harvey OX600 (R.J. Harvey Instrument, Hillsdale, NJ) as described by Thompson et al. (1999). The produced $^{14}CO_2$ was trapped in 10 mL of ^{14}C Harvey scintillation cocktail. A Beckman liquid scintillation counter LS 6000IC (Beckman Coulter, Fullerton, CA) was used for analysis.

Phosphor images were analyzed to determine signal intensity and distribution of radioactivity using ImageJ® image analysis software. Signal intensity was based on gray value of selected pixels, corrected for background, and multiplied by the selected area. Standard errors are derived from analytical triplicates to account for error and variability in the selection process.

Biodegradation of TNT, RDX, and HMX in Unplanted Soils

Soil used in the experiment was unplanted and either contaminated immediately prior to incubation or was contaminated and aged for 18 months while kept at 4°C. The experimental setup for freshly contaminated soil consisted of soil which was: sterile and kept in the light, sterile and kept in the dark, non-sterile and kept in the light, or non-sterile and kept in the dark. The experimental setup for soil which was contaminated and aged for 18 months consisted of soil which was non-sterile and either kept in the light or dark.

Experiments were carried out in pint-sized glass jars. The experiment was conducted at 30°C under a 16:8 hour light:dark photoperiod (150 μmol s^{-1} m^{-2}). Jars kept in the dark were covered with aluminum foil, loosely enough to allow for passive gas exchange. The soil was kept moist with deionized, sterile water, but was not saturated to keep soil conditions aerobic. At each time step in the experiment, samples were taken from eight locations in each jar and composited to account for heterogeneity. All experiments were conducted in triplicate.

TNT and analytical standards were purchased through Chemservice (Westchester, PA). RDX was synthesized in-house according to (Ampleman et al., 1995) and purified by recrystallization. Synthesized RDX was 99% pure or higher as indicated by High Performance Liquid Chromatography (HPLC) analysis. HMX was synthesized in-house according to (Ampleman et al., 1999), and determined to be 99% pure or higher as indicated by HPLC and Neutron Magnetic Resonance (NMR) analysis.

Soil was sterilized through gamma irradiation performed by Sterix Isometrics. Both soil types underwent gamma irradiation using ^{60}Cobalt at 30 – 40 kGy. Following sterilization, samples of each soil type were diluted in minimal salts media, spread onto full strength tryptic soy broth agar plates, and allowed to incubate for several weeks in the dark at 30°C. No bacterial colonies formed during the incubation.

Soil to be aged was contaminated separately with TNT and RDX to a nominal concentration of 100 mg/kg and 50 mg/kg, respectively and then stored for 18 months at 4°C prior to the start of the experiment. Freshly contaminated soil was contaminated with TNT, RDX, and HMX to a nominal concentration of 100 mg/kg. Explosives dissolved in acetonitrile were first mixed vigorously into 6% of soil mass, subsequently blended with the remaining soil for 20 seconds, mixed thoroughly, and then blended again for 20 seconds to obtain a homogeneous contamination. Soils were stored at 4°C until use in degradation experiments.

Extraction of explosives from soil was performed using a modified version of EPA Method 8330B (USEPA, 2006). Soil samples were dried at room temperature until a constant weight was reached. Each sample was then ground with mortar and pestle, placed in a 15 ml vial, and extracted with 10 ml acetonitrile to 1 g soil in an ultrasonic bath for 18 hours. The samples were then filtered under 0.20 µm Durapore membrane filters. Sample filtrate was then collected for analysis by HPLC.

Analyses of TNT, TNT metabolites, RDX, and HMX were performed by HPLC (HP Series 1100; Hewlett-Packard, Palo Alto, CA) using a C_{18} Supelcosil® LC-18 column (Supelco, Bellefonte, PA). The mobile phase consisted of acetonitrile:deionized water 50:50 v/v at a flow rate of 1.0 ml/min. Compounds were detected by UV absorbance at 230 nm and 254 nm using a UV visible photodiode array detector (HP series 1100). Calibration standards and blanks were analyzed before and after sample runs to ensure quality control.

Phytoremediation of RDX in Planted Soils

The laboratory study consisted of five microcosms using native Lakeland soil from Eglin AFB planted with either bahiagrass (*Paspalum notatum*) Pensacola, the excised roots of bahiagrass Pensacola, hybrid poplar (*Populus deltoides x nigra,* DN34), the excised roots of poplar, or remained unplanted as a control. The study included triplicates of each microcosm. The experiment was conducted for 56 days with soil samplings at days 0, 14, 28, and 56. On day 56, the plants were sacrificed. The leaves and roots of the poplars and the blades and roots of the bahiagrass were excised for analysis. The roots excised at the start of the experiment were extracted and analyzed. All roots were rinsed thoroughly of soil prior to processing.

The triplicates of each microcosm were planted in individual 4-inch diameter by 3 ¾-inch high Panterra® plastic planting pots (Greenhouse Megastore, Danville, IL). The planting pots had holes in the bottom to allow excess water to drain in order to keep the soil aerobic. Tin foil was placed over each pot in order to prevent phototrophic organisms from growing on the surface of the soil. The experiment was conducted in an environmental growth chamber at 30 ºC under a 16:8 hour photoperiod (150 µmol s^{-1} m^{-2}). The soil was watered periodically with 0.1x

Hoagland solution to maintain moisture and to provide nutrients for plants. Special care was given to not water to the point where water drained from the pots so as to ensure the contaminant did not migrate from the system.

Analytical standards were purchased through AccuStandard® (New Haven, CT) at a concentration of 1 mg/mL. RDX was synthesized in-house according to (Ampleman et al., 1995) and purified by recrystallization. Synthesized RDX was 99% pure or higher according to analysis by HPLC.

The soil was freshly contaminated with RDX to a concentration of 25 mg/kg. Approximately 400 g of soil was used in each pot. In order to achieve homogenous soil contamination, 100 g of soil was contaminated with RDX dissolved in acetonitrile and was then well mixed. Additional mass was added and well mixed until the necessary mass of soil was achieved. The soil was then immediately used for the experiment.

For each sampling, eight samples from each pot were taken for the entire depth of soil to account for heterogeneity. The samples were homogenized by mixing for approximately 2 minutes. The extraction of explosives from soil was performed according to a modified version of EPA Method 8330B (USEPA, 2006). The soil was left to dry at room temperature until a constant weight was achieved. A mortar and pestle were used to crush the soil into fine grains. 2 g of soil was placed in a 15 mL vial with 10 mL of acetonitrile for the extraction of the energetic compounds. The vials were then placed in an ultrasonic bath for 18 hours. Samples were filtered with 0.20 μm Durapore© membrane filters. The sample filtrate was then used for analysis.

The extraction of explosives from plant tissue was performed according to a modified version of EPA Method 8330B (USEPA, 2006). Following the excision of the roots and leaves or blades, a sample of each tissue was crushed and homogenized using a mortar, pestle, and liquid nitrogen. 2 g of plant tissue was placed in a 15 mL vial with 10 mL of acetonitrile for the extraction of the energetic compounds. The vials were then placed in a refrigerated ultrasonic bath for 18 hours. Samples were filtered with 0.20 μm Durapore© membrane filters. The sample filtrate was then used for analysis.

The explosives extracted from soil were analyzed using HPLC (HP Series 1100; Hewlett-Packard, Palo Alto, CA) using an Acclaim® Explosives E1 column (Dionex Corporation). The samples were analyzed with a mobile phase of methanol:deionized water 43:57 v/v at a flow rate of 1.0 mL/min. Detections were measured at a UV absorbance of 230 nm and 254 nm using a UV visible photodiode array detector (HP Series 1100).

Explosives extracted from plant samples were analyzed using liquid chromatography-mass spectrometry (LC/MS). An Agilent 6140 Quadrupole LC/MS was used with an Acclamin 120 Å C_{18} column (2.1 x 150 mm, 3μm; Dionex Corporation). The mass spectrometer was operated in negative-ion electrospray mode. A mobile phase of acetonitrile:2mM ammonium acetate 50:50 v/v at a flow rate of 0.4 mL/min.

Calibration curves were constructed using standards before and after each sample run for quality control. Standards were also placed after every ten sample vials in order to verify retention times and elution order. The standards, EPA 8330-R Explosives Mix, were ordered from AccuStandard® (New Haven, CT). The explosives mix included the following components: 1,3-dinitrobenzene (DNB), 1,3,5-trinitrobenzene (TNB), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), 4-nitrotoluene (4-NT), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene

(2,6-DNT), nitrobenzene (NB), tetryl, TNT, RDX, and HMX. The standards were used to identify and quantify explosives and metabolites in samples.

Sorption Analyses

Ring-U-^{14}C and unlabeled organic compounds were purchased from Sigma-Aldrich Chemical Co, and used without further purification. All sorption isotherms were obtained using a batch equilibration technique at room temperature (23 ± 1°C) in screw cap vials (4 mL) with aluminum foil liners. The background solution was comprised of 0.01 M $CaCl_2$ in deionized distilled water with 200 mg/L NaN_3 as a biocide (pH=7). Initial concentrations ranged from 0.035-19.8 mg/L for TNT. Due to low aqueous solubility, stock TNT solutions were made at high concentrations in acetonitrile (AcCN) before being added to the background solution. The ^{14}C-labeled organic compound and its non-radioactive aqueous solutions were mixed with the solid samples (e.g., muck soil, sandy soil, poplar root, poplar leaf) at different solid to solution ratios. The ratios were adjusted to achieve 30 to 80% sorption of TNT at apparent equilibrium. AcCN concentrations were always less than 0.1% of the total solution volume to avoid co-solvent effect. Isotherms consisted of 9 concentration points; each point, including the blank, was run in duplicate. The vials were sealed with aluminum foil-lined Teflon screw caps and then placed on a shaker for 3 days at room temperature (23 ± 1°C). Preliminary tests indicated that apparent equilibrium was reached before 2 days. The vials were then centrifuged at 4500 rpm for 30 min, followed by 2.0 mL removal of supernatant, which was added to a Scintiverse cocktail (10 mL) for scintillation counting (Bechman LS6500). Because of little sorption by vials and no biodegradation, sorbed sorbate by the sorbents was calculated by mass difference.

Microarray Analyses for Gene Expression in Hybrid Poplar

Minimum Information About a Microarray Experiment (MIAME) guidelines were consulted with respect to Affymetrix® designed experiments. MIAME reporting is primarily geared towards spotted arrays and not the manufactured arrays used in this experiment. However, we have used the MIAME guidelines were applicable.

Poplar tree cuttings (*Populus deltoides x nigra,* DN34) were obtained from Segal Ranch Greenhouse (Grandview, WA) and kept in a 4°C cooler to maintain dormancy until needed. Cuttings were grown in a modified Hoagland's solution under a 16 hour light and 8 hour dark diurnal cycle. The growth solution was replaced twice a week for approximately eight weeks when plant shoots and roots reached sufficient size for experimentation.

Cuttings were transferred to foil wrapped 500 mL flasks with modified Hoagland's Solution. Treatment flasks contained Hoagland's with a concentration of 5 mg TNT L^{-1} (2.2 x 10^{-5} M) dissolved in acetonitrile (Sigma-St. Louis) and negative control flasks contained the Hoagland's solution and acetonitrile. Control plants were sacrificed at time zero with triplicate plants sacrificed at 8, 24, and 48 hours after exposure to TNT. These sampling times were pre-determined in our lab by several experiments specifically conducted to find the optimum sampling times for the course of exposure in this system. The optimal sampling time was found to be at 8 hours, when TNT had just begun to be removed from the aqueous solution, at 24 hours, when approximately half of the initial concentration remained in the aqueous solution, and at 48 hours, when all of the original TNT concentration had been removed from the aqueous solution.

RNA extraction took place under a hood specifically used for this purpose. All instruments, tubes, surfaces, containers, and gloves were sprayed or wiped down with RNase *AWAY*™ (Invitrogen). All plant cuttings chosen for extraction were taken from solution and the

roots were immediately and fully immersed in liquid nitrogen. The frozen roots were then broken off into a mortar containing liquid nitrogen and ground with a pestle. Approximately 150 mg of crushed and frozen root material was then placed in a tube containing 450 μL of RNAlater® (Ambion) and vortexed to allow the stabilizer to permeate the entire sample. Small (1.0 mm) zirconia/silicon beads were added and the tubes were shaken on a bead beater for maximum cellular disruption. Total RNA extraction was completed using the RNeasy® Plant Mini Kits from Qiagen.

The *Populus* genome microarrays were purchased from Affymetrix® through the University of Iowa DNA Facility. The array design is a synthesized oligonucleotide array on a glass plate. Individual spot identification is proprietary information and is available only through Affymetrix®. Extracted RNA concentration and quality was determined using the Agilent Bioanalyzer® at the DNA Facility. Samples met the facility guidelines of an A_{260}/A_{280} ratio between 1.9 and 2.1 and a minimum of 10 μg of total RNA. Gel analysis showed distinctive 28S and 18S peaks with little to no degradation. Hybridization of the samples to the GeneChips® was done at the DNA facility using a model 250 GeneChip® Fluidics Station.

Affymetrix® microarray files were uploaded and analyzed using ArrayAssist® and a time course package "expression and analysis of differential gene expression (EDGE)" designed to run on the R statistical program platform (Leek et al., 2006; Storey et al., 2005). Probe level analyses and quality control checks for the GeneChips® were performed and no abnormalities noted. Data was transformed using PLIER and log transformations. Data was normalized over the four time points and among the triplicate samples to the probe control responses. Triplicate columns were combined and means and standard deviations were calculated.

Statistically significant differences between the controls and three treatments at different sampling times were reported when a greater than (or less than) 2-fold change and a P-value of less than 0.05 was observed. Approximately 2305 genes met these criteria using the ArrayAssist® program. The R "EDGE" program analysis resulted in 9327 statistically significant genes. An overlap analysis was performed that resulted in 1443 genes, which were identified as significant in both statistical programs. An individual analysis of these genes was undertaken.

T-RFLP to Characterize of Microbial Communities in Soil

T-RFLP is a culture-independent method of obtaining the genetic fingerprint of a microbial community. During the T-RFLP method, extracted DNA is amplified through Polymerase Chain Reaction (PCR) using a fluorescent primer. The fluorescent molecule attached to the primer is tagged to one end of the PCR amplicons during the PCR process. The amplified PCR product is then digested using restriction enzymes, producing terminal restriction fragments (fragments which are tagged with a fluorescent molecule at their terminal end). The terminal restriction fragments (T-RFs) are then separated by electrophoresis providing their size in base pairs and intensity of fluorescence. T-RF sizes can then be compared to known sequences in databases for phylogenetic assignment and analysis of microbial community (Blackwood, Marsh, Kim, & Paul, 2003).

T-RFLP has been used successfully for the comparison of bacterial diversity and composition in environmental soil samples (Hackl, Zechmeister-Boltenstern, Bodrossy, & Sessitsch, 2004). T-RFLP has also been implemented for monitoring the spatial and temporal variations in the microbial structure of agricultural soil (Lukow, Dunfield, & Liesack, 2000).

This relatively new technique may be an important addition to more completely characterizing remediation efforts at contaminated sites.

Soil DNA was extracted from 0.5 g of bulk soil for both Lakeland Soil and Dorovan Muck using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to kit instructions. DNA concentrations following extraction ranged from approximately 2 – 40 μg/ml.

The PCR method used is similar to the protocol described by Kent et al. (2003). PCR amplification of soil DNA was performed using a HotStar Taq® Master Mix Kit (Qiagen) on a Mastercycler Gradient® thermocycler (Eppendorf, Hamburg, Germany) using the following program: a 15 minute start at 94°C, followed by 35 cycles consisting of denaturation (35 s at 94°C), annealing (45 s at 55°C), and extension (90 s at 72°C), and a final extension for 2 min at 72°C. Reaction mixtures for PCR contained 50 μl HotStar Taq® Master Mix, 1 μl of each primer, 1 μl of DNA extract, and 2 μl of BSA buffer in a final volume of 100 μl. The primers used were 8F and 1492R. PCR products were then purified using a QIAquick® PCR Purification Kit (Qiagen) according to kit instructions.

PCR products were digested separately with the restriction enzymes Hha1, Msp1, and Rsa1. Multiple digests using the three restriction enzymes were carried out to increase the specificity of the phylogenetic assignments. The lengths of the terminal restriction fragments were determined by electrophoresis with a Model 3730 DNA Analyzer (Applied Biosystems, Inc.). The mixture which was analyzed contained 1 μl of digested PCR product, 9.5 μl HiDi Formamide, and 0.5 μl of DNA fragment length standard (GeneScan 1200 LIZ). Negative controls were processed through the entire method and showed that there was no contamination during any of the procedures.

Data from DNA fragment sequencing were analyzed using PeakScanner 1.0 software (Applied Biosystems, Inc.). Data tables containing the fragment size and abundance data for each digest were exported from PeakScanner as text files. The resulting text files were then uploaded to the T-RFLP phylogenetic assignment tool (PAT) provided by the University of Wisconsin Center for Limnology (Kent, et al., 2003). The PAT output data were then analyzed to determine the relative abundance for each phylogenetic assignment. The phylum, order, class, and family for each phylogenetic assignment were determined using the National Center for Biotechnology Information (NCBI) Nucleotide database.

Field Soil and Plant Sampling at Eglin AFB

Sampling was conducted on each plot according to the systematic random sampling method. The strategy and design behind this method is to obtain soil sample increments positioned at collection points that are distributed relatively evenly throughout the sampling area (Hewitt et al., 2007). This was necessary due to the heterogeneity associated with explosive contamination on military ranges (Jenkins et al., 2006). This method, applied to the plots at Eglin AFB, results in 100 discrete soil and plant samples from three separate passes over each plot in the planted region as shown in Figure 2. In addition, 40 discrete soil samples were obtained from a perimeter offset 12 feet from the edge of the planted region every 15 feet in order to serve as an unplanted control. During the May 26-27, 2009 sampling, the sampling method was applied to all three plots resulting in 140 soil samples and 100 plant samples per plot. In all subsequent samplings, only plot #1 was sampled to the full extent because of the small number of detections at the other two plots. Due to time constraints and lack of detections, samples were pared to only 34 discrete soil and plant samples for plots #2 and #3 along with an

additional 14 discrete soil samples taken every 45 feet around the perimeter. The samples collected at plots #2 and #3 were still sampled according to the systematic random sampling method. As seen in Figure 2, only the first pass was completed.

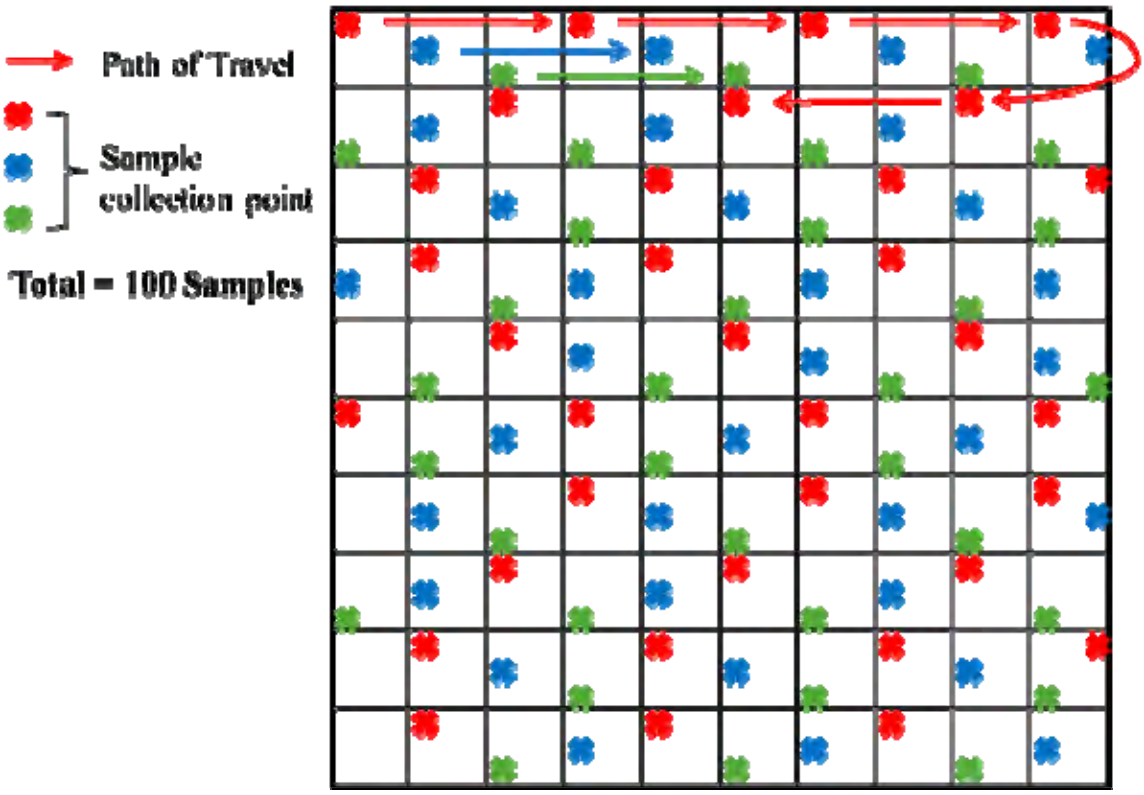


Figure 2. Systematic random sampling method used for plots #1-3 (Jenkins et al., 2006). The entire 100 samples were taken at Plots #2 and #3 for only the May 26-27, 2009 sampling. Each subsequent sampling only the first 34 were retrieved.

The May 26-27, 2009 sampling was immediately prior to the planting of the Bahiagrass Pensacola. Therefore, the May 26-27, 2009 sampling should be considered the "time equals zero" sampling. Subsequent sampling occurred at 6 months (November 18-19, 2009), 12 months (May 24-25, 2010), and 18 months (November 13-14, 2010). The four samplings resulted in 1,111 soil samples and 487 plant samples which were used to characterize each plot over the course of the study.

The soil samples were retrieved using a 2 cm diameter steel soil corer at a depth of 5 cm and were then placed in a re-sealable plastic bag. As shown in Figure 3, the soil cores were actually taken at a depth of 7 cm, but the top 2 cm was removed to avoid sampling the sod in which the Bahiagrass was initially established. The steel soil corer was wiped down with paper towels between samplings in order to minimize cross-contamination. Pruning shears were utilized to take cuttings of the Bahiagrass at each soil sampling location in the planted region of each plot.

Figure 3. Soil corer used to collect Lakeland soil samples at the Range C-62 site on November 13, 2010.

Field Soil and Plant Sample Analyses

The extraction of explosives from soil was performed according to a modified version of EPA Method 8330B (USEPA, 2006). The soil was left to dry at room temperature until a constant weight was achieved. A mortar and pestle were used to crush the soil into fine grains. Two grams of soil was placed in a 15 mL vial with 10 mL of acetonitrile for the extraction of the energetic compounds. The vials were then placed in an ultrasonic bath for 18 hours. Samples were filtered with 0.20 µm Durapore© membrane filters. The sample filtrate was then used for analysis.

The extraction of explosives from plant tissue was performed according to a modified version of EPA Method 8330B (USEPA, 2006). A sample of plant material was crushed and homogenized using a mortar, pestle, and liquid nitrogen. Two grams of plant material was placed in a 15 mL vial with 10 mL of acetonitrile for the extraction of the energetic compounds. The vials were then placed in an ultrasonic bath for 18 hours. Samples were filtered with 0.20 µm Durapore© membrane filters. The sample filtrate was used for analysis.

The explosives extracted from soil were analyzed using HPLC (HP Series 1100; Hewlett-Packard, Palo Alto, CA) using an Acclaim® Explosives E1 column (Dionex Corporation). The samples were analyzed with a mobile phase of methanol:deionized water 43:57 v/v at a flow rate of 1.0 mL/min. Detections were measured at a UV absorbance of 230 nm and 254 nm using a UV visible photodiode array detector (HP Series 1100).

Explosives extracted from plant samples were analyzed using liquid chromatography-mass spectrometry (LC/MS). An Acclaim Explosives E2 column (2.1 x 150 mm, 3µm; Dionex Corporation) was used on an Agilent 6140 Quadrupole LC/MS. The mass spectrometer was

operated in negative-ion electrospray mode. A mobile phase of 2mM ammonium acetate in methanol:deionized water 48:52 v/v at a flow rate of 0.3 mL/min.

In addition to analysis using HPLC, soil samples from May 24-25, 2010 and November 13-14, 2010 were analyzed with LC/MS. This was a quality assurance measure to verify the detections and concentrations found using HPLC.

Calibration curves were constructed using standards before and after each batch of samples were run in order to ensure quality. Standards were also placed after every ten sample vials in order to verify retention times and elution order. The standards, EPA 8330-R Explosives Mix, was ordered from AccuStandard (New Haven, CT). The explosives mix included the following components: 1,3-dinitrobenzene (DNB), 1,3,5-trinitrobenzene (TNB), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), 4-nitrotoluene (4-NT), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), nitrobenzene (NB), tetryl, TNT, RDX, and HMX. The standards were used to identify and quantify explosives in samples. While HPLC was able to detect all of the explosives in the mixture, the method used for analysis with LC/MS was only able to detect TNT, RDX, HMX, 2-ADNT, 4-ADNT, 2,4-DNT, 2,6-DNT, TNB, and Tetryl.

V. Results and Discussion

Task 1. Sampling and Site Selection

Contact was made with Eglin Air Force Base (Eglin AFB) in Florida and a site visit was conducted August 21-22, 2006 in order to scout potential sites for soil collection and the implementation of the phytoremediation field study. Coordinators for our visit at Eglin Air Force Base included Amanda Stevens, Fire Ecologist, and Sandy Pizzolato, Erosion Ecologist. Mr. Pizzolato acted as a guide around the base, provided an overview of the ecology of the site, and aided in collection of soil and native plant specimen samples.

Soil samples were collected during the August 21-22, 2006 field site visit at Eglin AFB. 136.2 kg of Lakeland Sandy Soil was collected at GPS coordinates -86:38:41.053587, 30:35:31.284834 and 63.56 kg of Dorovan Muck Soil was collected at GPS coordinates -86:39:41.424052, 30:35:26.098330. The locations of the collection sites are shown in Figure 4. Soil samples were shipped in 28-quart coolers (4 coolers per soil type) at 4-8 °C from Niceville, FL to The University of Iowa Department of Civil and Environmental Engineering in Iowa City, IA. The soils were stored at 4 °C.

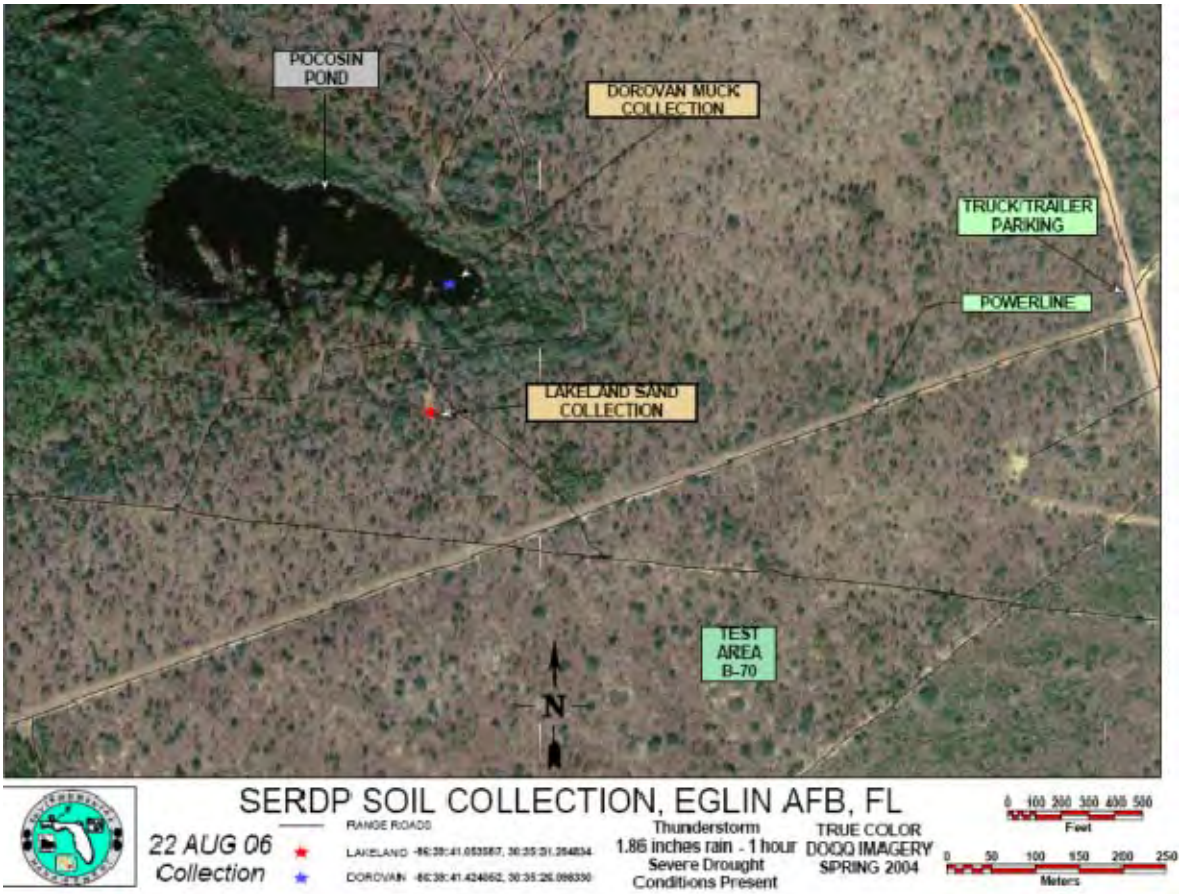


Figure 4. A map of a portion of Eglin AFB showing soil collection sites for both Lakeland Soil and Dorovan muck. Credit: Sandy Pizzolato.

A second trip to Eglin AFB to collect more soil samples was completed March 1, 2007. Lakeland soil was collected in the same location as on the previous visit. The previous collection site for Dorovan muck was underneath 2 feet of standing water and could not be resampled. A

secondary site, Anderson Pond (GPS coordinates: -86:30:55.614, 30:33:34.746), was used for collection of Dorovan muck. The soil is underwent physical-chemical characterization to confirm that the soil is the same.

Samples of two native trees, adapted to local climate conditions, were collected: *Cliftonia monophylla* and *Cyrilla racemiflora* (Titi) during a field site visit. Additionally, a list of native plants used previously on site as well as a seed provider (Adams-Briscoe Seed Co., Jackson, GA) for local grasses and legume species were suggested. See Table 2 for list of suggested species.

Table 2. Grass and legume species native to Eglin Air Force Base.

Species	Common Name
Cassia fasiculata	Common Showy Partridge Pea
Panicum vigratus	Switchgrass (Alamo)
Lespedeza striata	Lespedeza (Kobe)
Lespedeza serica	Lespedeza (Serala)
Paspalum notatum	Bahiagrass Pensacola
Andropogon virginicus	Broomsedge

The soil types collected at Eglin Air Force Base included Lakeland soil and Dorovan muck. Lakeland soil is the predominant soil type at Eglin AFB. It is classified as a sandy soil. Dorovan muck is less common and found in locations such as swamp and stream beds. Samples of two collected soil types were sent to A&L Analytical Laboratories in Memphis, TN for nutrient, pH, bulk density, and texture analyses. The results of these analyses are summarized below in Table 3 and Table 4. The Lakeland soil is a very sandy soil with small (2-5 cm) scattered clay lenses while the Dorovan muck is an organic-rich clay soil. Both soils are acidic with a low nutrient content in general, but are especially low in phophorus and somewhat low in nitrogen.

Table 3. Summary of physical-chemical soil characteristics.

	Dorovan Muck	Lakeland Soil
Soil pH	4.1	5.1
Buffer pH	6.14	6.93
Bulk density (g/cc)	0.94	1.45
Phosphorus (lb/acre)	22 (low)	16 (low)
Potassium (lb/acre)	40 (very low)	36 (very low)
Calcium (lb/acre)	1018 (high)	512 (med)
Magnesium (lb/acre)	182 (med)	38 (low)
Nitrate nitrogen (lb/acre)	16	14
Ammonical nitrogen (lb/acre)	20	0
CEC (meq/100g)	10.6	1.9
Organic matter content (%)	4.8	0.4

*CEC = cation exchange capacity

Table 4. Results of soil texture analysis.

	Dorovan Muck	Lakeland Soil
Sand (%)	11.1	70
Silt (%)	16.7	4
Clay (%)	72.2	26
Classification	Clay	Sandy clay loam

Both collected soil types, Dorovan muck and Lakeland soil, were used for aging experiments and pot studies. Growth protocol studies for plants in the lab were conducted for *Panicum vigratum* (switchgrass) Alamo, *Cassia fasiculata* (partridge pea), *Paspalum notatum* (bahiagrass) Pensacoloa, *Populus trichocarpa*, *Cliftonia monophylla* (Titi tree), and *Cyrillia racemiflora* (Titi tree). Of these plants, three successful candidates were *P. vigratum* and *P. notatum*, to be used as a native grass mixture for testing ranges, and *P. trichocarpa*, a tree species with a wide climate range that may be applicable along stream banks in Eglin AFB. These plants were used in potted plant studies.

Task 2. Plant Growth Protocols

Preliminary pot studies and hydroponic growing studies were performed to develop appropriate growth procedures for native plant species in laboratory and greenhouse settings. Pot studies compared the use of soils in fertilized and unfertilized conditions to help establish requirements for growth of selected plant species on site and during future experiments. Hydroponic studies established a method for growing plants under "clean" conditions for phytotoxicity and uptake studies.

Procedures for measuring biodegradation were established. In degradation studies, plants were exposed to radiolabeled explosives or propellant in their growth medium in a sealed plant chamber with an air inlet and outlet with both hydroponic and soil systems. The outlet was equipped with CO_2 traps and an organic trap to track potentially volatilized metabolites. At the end of the exposure period, the solution or soil and plant tissues were extracted and analyzed for

radioactivity using a biooxidizer (for plant tissues) and scintillation counter. Liquid chromatography/mass spectrometry was also used to determine metabolites from soil and plant tissue. Phosphor imager autoradiography was used to monitor uptake of radiolabeled explosives by plants and determine where the explosive and/or its metabolites are transported and accumulate within the plant.

Radiolabeled TNT and RDX were added and aged 4-6 months at 4°C prior to planting with native grass mixture. Each soil type was contaminated separately with [U-^{14}C]-RDX to a final concentration of 50 mg/kg and [U-^{14}C]-TNT to a final concentration of 100 mg/kg. The final activity of the labeled contaminants in the soil was 11,100 DPM/1g soil. The final volume of contaminated soil was 28 L for each soil type and explosive, or 40.6 kg of Lakeland soil and 26.3 kg of Dorovan muck.

Task 3. Biodegradation Experiments

Phytotoxicity and Biodegradation of TNT by Grass Mixture and Hybrid Poplar

A microcosm study was conducted using potted plants. The plantings included a grass mixture of bahiagrass (*Paspalum notatum*) Pensacola and switchgrass (*Panicum vigratum*) Alamo, and hybrid poplar cuttings (*Populus deltoides x nigra*, DN34) using two different soils collected from Eglin AFB, Dorovan muck and Lakeland Soil. The soils were spiked with 2,4,6-trinitrotoluene (TNT) and the experiment was carried out in a controlled environmental growth chamber. The purpose of the microcosm study was to evaluate the performance of the planted systems in removing TNT from contaminated soils. Soils were freshly contaminated with 100 mg/kg TNT and 5 μCi/kg [U-^{14}C]-TNT in the laboratory. This was done using the same process used to contaminate soils that were subsequently aged. Triplicates of the following conditions were tested for comparison: aged TNT-contaminated Dorovan muck (ADM) planted with grass mixture (10 g grass initially per pot), ADM planted with poplar cuttings (1 healthy plant per pot), freshly TNT-contaminated Dorovan muck (FDM) planted with grass mixtures, FDM planted with poplar cutting, unplanted controls for both ADM and FDM, aged TNT-contaminated Lakeland Soil (ALS) planted with grass mixture, ALS planted with poplar cuttings, freshly TNT-contaminated Lakeland Soil (FLS) planted with grass mixture, FLS planted with poplar cuttings, and unplanted controls for both ALS and FLS.

Additionally, hydroponically grown grasses were used to assess phytotoxicity and uptake of TNT. The grasses were initially spike with concentrations of 0, 2, 5, 10, and 25 mg/L TNT in 0.5x Hoagland solution and were placed in a controlled environmental growth chamber. Grasses were monitored for change in biomass, transpiration, and observable toxicity markers such as chlorosis or mortality.

Phytotoxicity of TNT on *Panicum vigratum* and *Paspalum notatum* was tested using hydroponically grown plants. Both grasses caused decreased concentrations of TNT in solution to 0 in 5 mg L^{-1} after 24 hours and 10 mg L^{-1} solutions after 48 hours (see Figure 5 and Figure 7). Change in biomass appeared to have affected *P. vigratum* at 10 mg L^{-1} (44μM) and in *P. notatum* at 25 mg L^{-1} (110 μM) (see Figure 6 and Figure 8). All exposed grasses eventually transpired all of the TNT initially provided in hydroponic systems. The high tolerance and high rate of uptake of TNT make these species promising for phytoremediation applications at Eglin AFB. Figure 9 provides an image of the phytotoxicity setup after 5 days exposure to TNT.

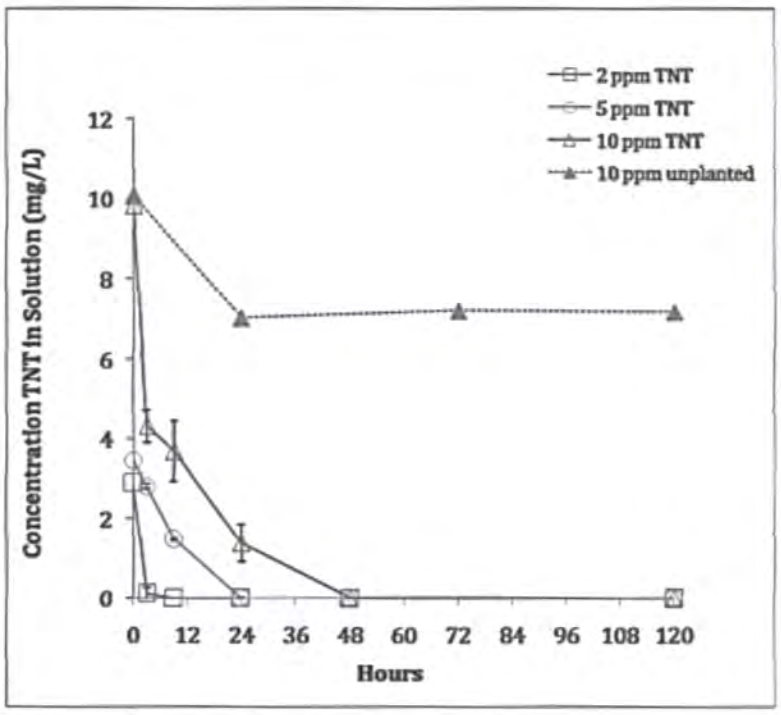


Figure 5. Removal of TNT from hydroponic solution by Panicum vigratum Alamo after spiking solution to give an initial concentration of 2, 5, or 10 mg L^{-1} TNT.

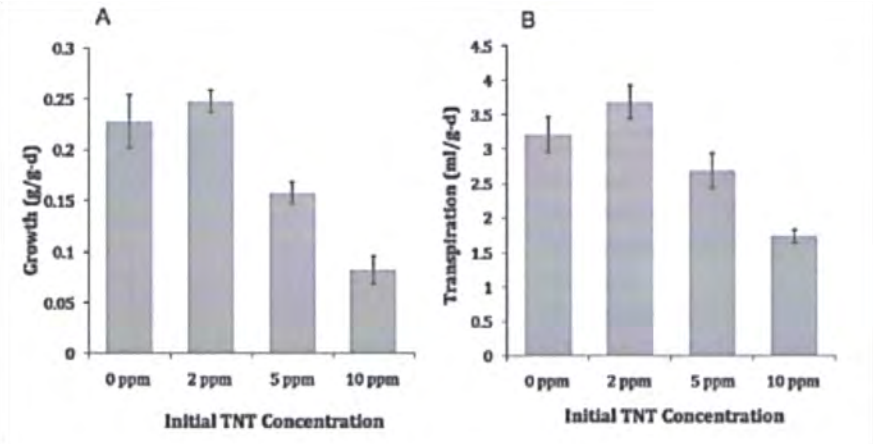


Figure 6. Measurements of TNT phytotoxicity in *Panicum vigratum* Alamo (switchgrass). (A) shows change in biomass after 5 days normalized to the initial biomass. (B) shows the differences in transpiration after 5 days, also normalized to the initial biomass.

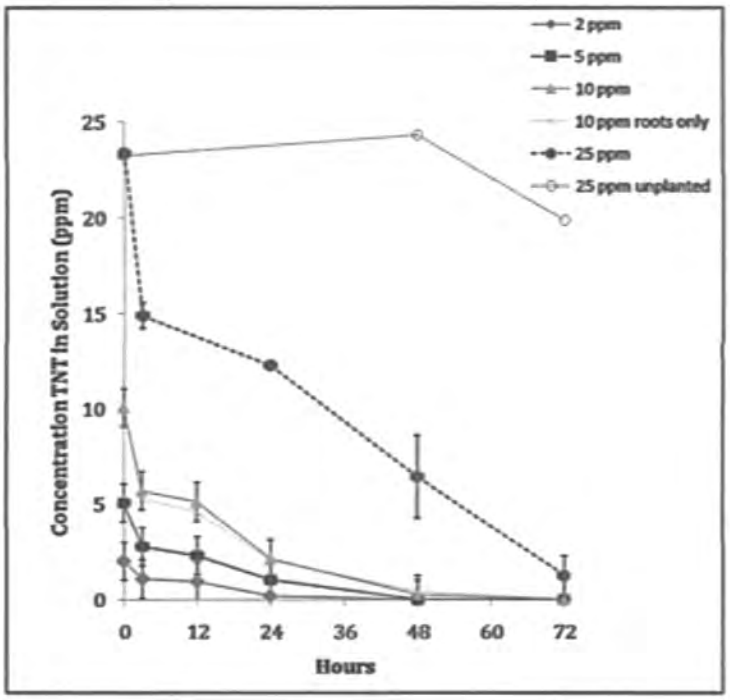


Figure 7. Removal of TNT from hydroponic solution by *Paspalum notatum* (bahiagrass) Pensacola after spiking solution to give an initial concentration of 2, 5, 10, 25 mg L^{-1} TNT.

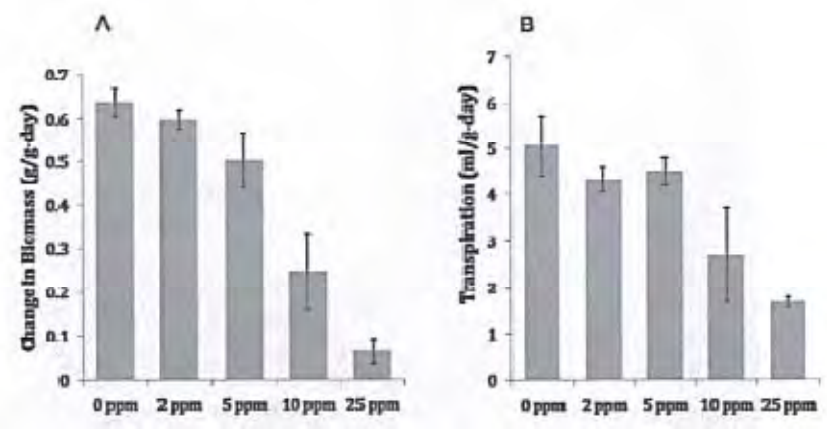


Figure 8. Measurements of TNT phytotoxicity in *Paspalum notatum* Pensacola (Bahiagrass). (A) shows change in biomass after 5 days normalized to the initial biomass. (B) shows the differences in transpiration after 5 days, also normalized to the initial biomass.

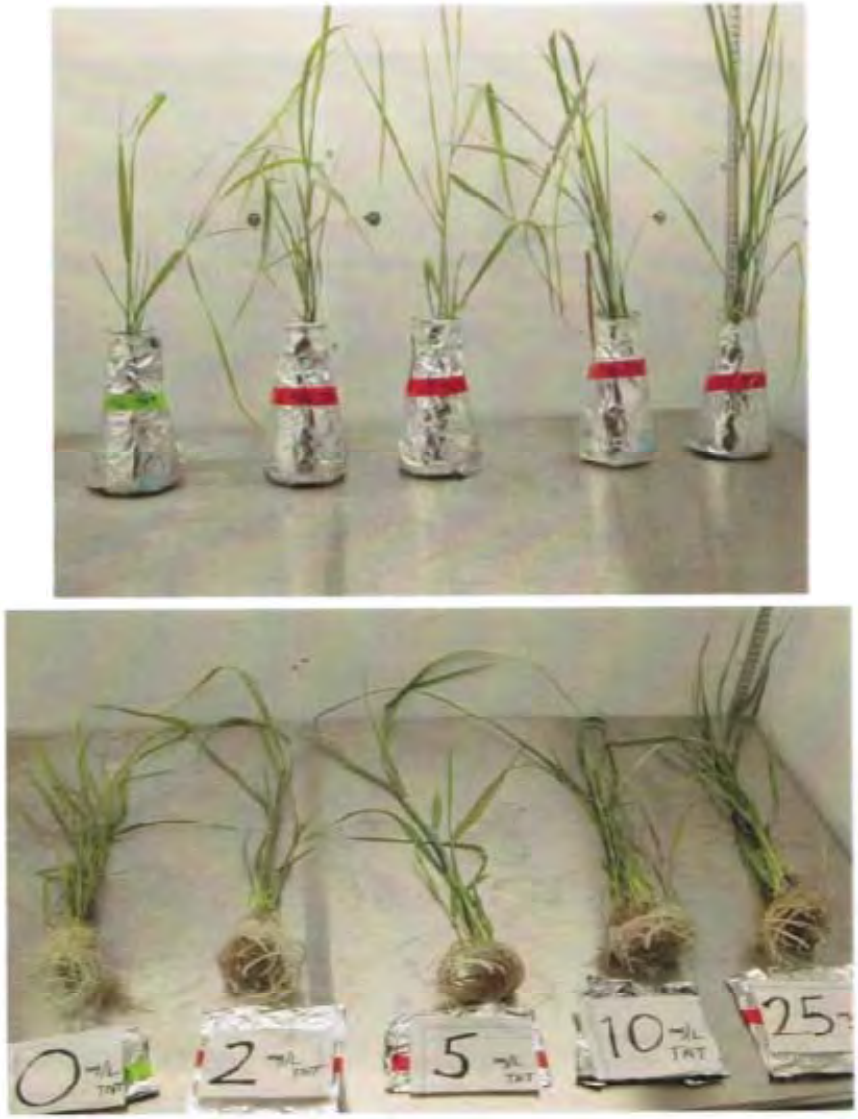


Figure 9. Experimental setup and switchgrass 5 days after exposure to 0, 2, 5, 10, and 25 mg L^{-1} TNT.

Figure 10 shows the experimental setup for the planted pot study in the environmental growth chamber. Soil type and age appear to have a greater impact on percent TNT removal than planting (see Figure 11 and Figure 12). In general, removal of TNT in Lakeland soils was more efficient than in Dorovan muck. The effects of aging were more significant in Dorovan muck, where aged soils retained higher concentration of TNT than freshly contaminated soils throughout the experiment. After 40 days, 100% removal of detectable, extractable TNT was observed in all freshly contaminated Dorovan muck plantings, 76% removal in aged Dorovan muck unplanted, 85% removal in aged Dorovan muck grass mixture plantings, and 83% removal in aged Dorovan muck poplar plantings. For Lakeland soils, aging had only a small effect on retention of TNT in soils. Greater than 100% removal was observed for all freshly contaminated Lakeland soil pots, as well as in the poplar planted aged Lakeland soil pot, after 40 days. In grass mixture planted aged Lakeland soil pots 96% removal was observed and in unplanted aged Lakeland soil 86% removal was observed. The poplars had to be replanted several times in

Lakeland soil, presumably because the trees are not as well suited for growth in sandy soil and the available concentration of TNT was much higher, reaching toxic levels of exposure, in Lakeland soil compared to Dorovan muck.

Figure 10. Experimental setup for microcosm study in an environmental growth chamber. Top: Dorovan muck soil treatment. Bottom: Lakeland soil treatments. There are four soil types: Freshly contaminated Dorovan muck, Dorovan muck aged with the contaminant for more than 6 months, freshly contaminated Lakeland soil, and Lakeland soil that has been aged with the contaminant for more than 6 months. Each soil type is planted with a poplar cutting, mixture of switchgrass and Bahiagrass, or is left unplanted. All treatments are in triplicate.

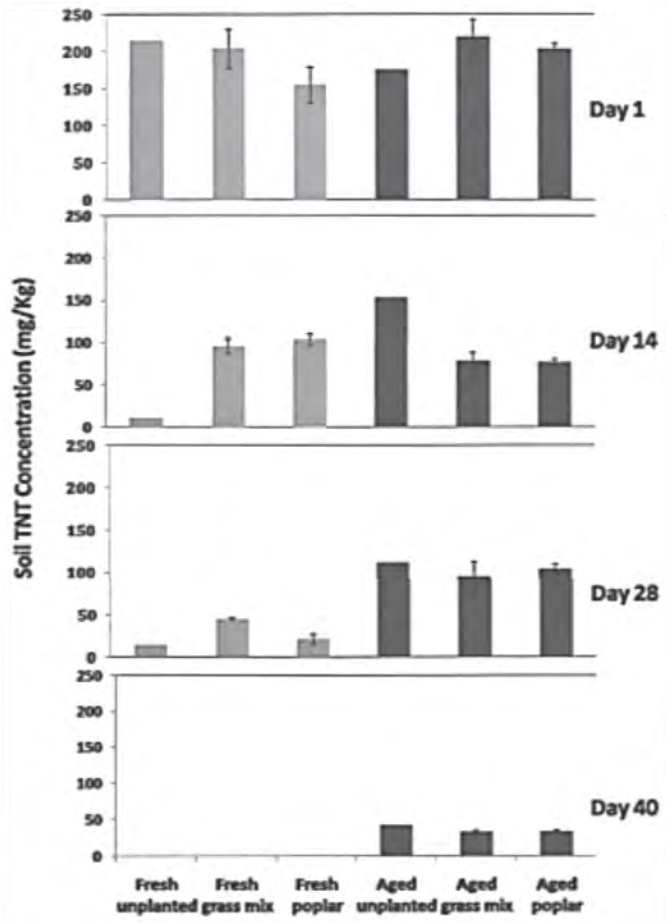


Figure 11. Concentration of TNT extractable from freshly contaminated Dorovan muck (light bars) and Dorovan muck that had been previously aged for 6 months with TNT (dark bars) at days 1, 14, 28, and 40 of the microcosm study. Plantings of a grass (switchgrass and Bahiagrass) mixture, poplar cutting, and unplanted controls were sampled with error bars representing standard error from samples of triplicate plantings.

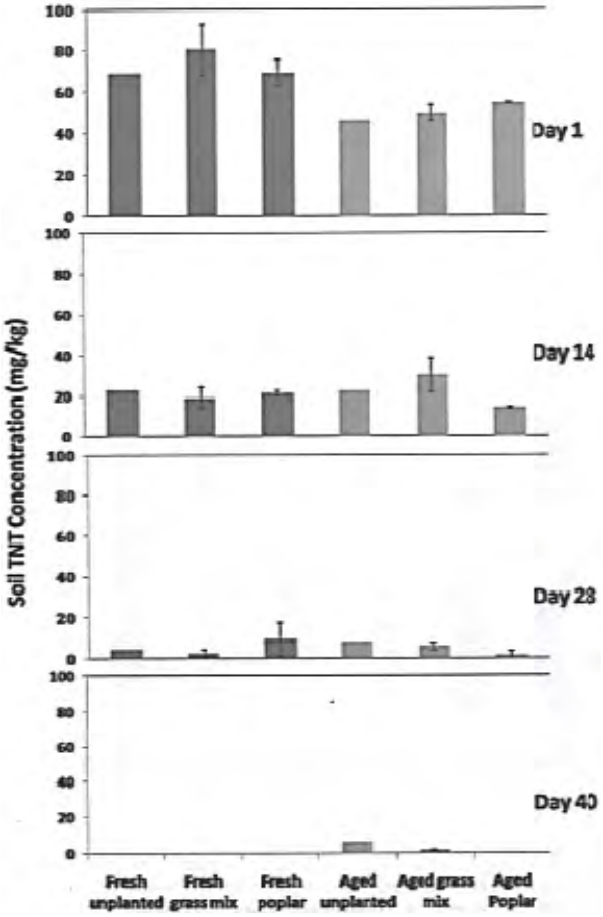


Figure 12. Concentration of TNT extractable from freshly contaminated Lakeland soil (dark bars) and Lakeland soil that had been previously aged for 6 months with TNT (light bars) at days 1, 14, 28, and 40 of the microcosm study. Plantings of a grass (switchgrass and Bahiagrass) mixture, poplar cutting, and unplanted controls were sampled with error bars representing standard error from samples of triplicate plantings.

Figure 13 and Figure 14 show the concentration of 2,4- and 4,6-aminodinitrotoluene (ADNTs), common metabolites of TNT, in soils over the course of the microcosm study. No ADNTs were detected in soils before day 14 in Dorovan muck pots and day 35 in Lakeland soil pots. ADNT concentrations were much higher in Dorovan muck soil than in Lakeland soil, reaching 78 mg/kg in unplanted freshly contaminated Dorovan muck compared with only 11 mg/kg in aged unplanted Lakeland soil. No ADNTs were detected in unplanted and grass planted freshly contaminated Lakeland soil. Of the freshly contaminated Lakeland soil pots, only the poplar treatment contained ADNTs at levels similar to aged Lakeland soil pots. Aged Dorovan muck pots had lower concentrations of ADNTs than freshly contaminated Dorovan muck, with 63-77 mg/kg in freshly contaminated pots and 47-53 mg/kg in aged pots. Among freshly contaminated Dorovan muck treatments, the unplanted pot had the highest concentration of ADNTs, followed by the grass, then poplar planted pots. In aged Dorovan muck the grass mixture had the highest concentration of ADNTs while the unplanted and poplar treatments had similar concentrations of ADNTs.

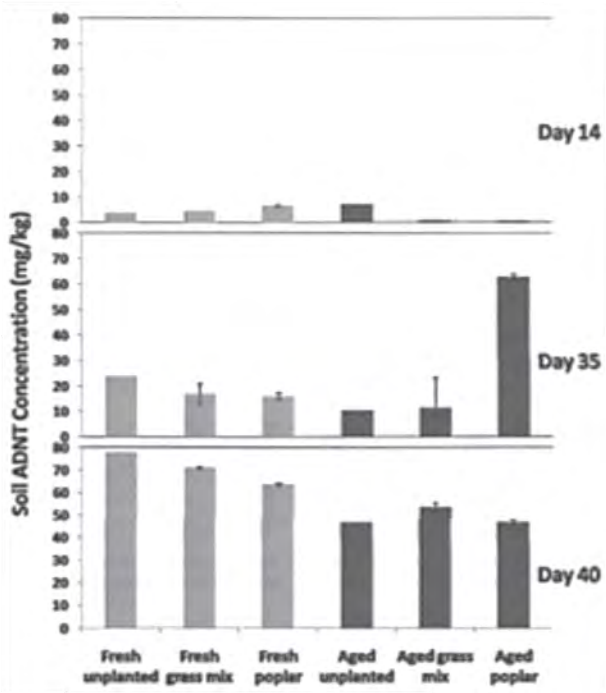


Figure 13. Concentration of ADNTs in freshly contaminated Dorovan muck (light bars) and Dorovan muck that had been previously aged for 6 months with TNT (dark bars) during the course of the microcosm study. Plantings of a grass mixture (switchgrass and Bahiagrass), poplar cutting, and unplanted controls were sampled with error bars representing standard error from samples of triplicate plantings.

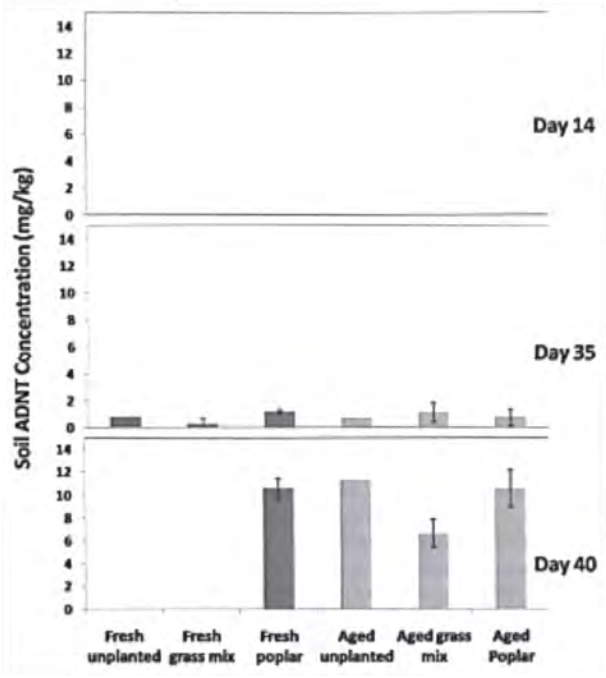


Figure 14. Concentration of ADNTs in freshly contaminated Lakeland soil (dark bars) and Lakeland soil that had been previously aged for 6 months with TNT (dark bars) during the course of the microcosm study. Plantings of a grass mixture (switchgrass and Bahiagrass), poplar cutting, and unplanted controls were sampled with error bars representing standard error from samples of triplicate plantings.

The percentages of total radioactivity, initially spiked in soils as [U-^{14}C]-TNT, remaining unextractable (by acetonitrile) soil residues are given in Figure 15 and Figure 16. Aging or planting does not appear to impact the unextractable TNT in DM soil, all values are >3% (see Figure 15). Freshly contaminated LS planted with poplar had the highest fraction of unextractable TNT in soils residues at 7%. Other LS pots had between 0 and 3% radioactivity remaining in extracted soils.

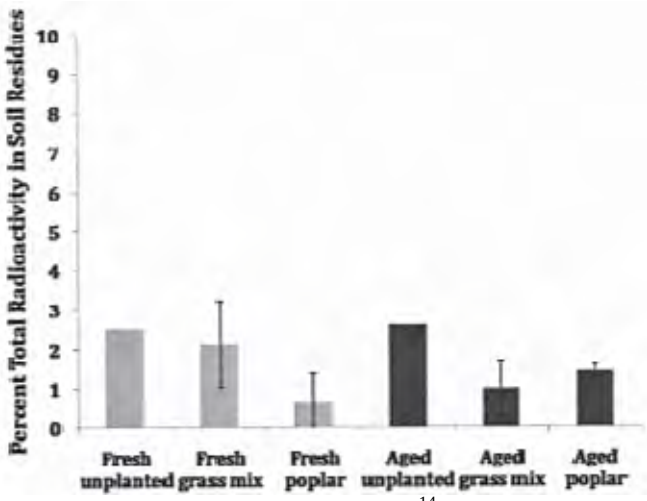


Figure 15. Percent of total radioactivity from [U-^{14}C]-TNT remaining in Dorovan muck soil residues following extraction by acetonitrile at the completion of the microcosm study. Soils were freshly contaminated with TNT at the beginning of the experiment (light bars) or aged with TNT for 6 months (dark bars) prior to the planting of a grass mixture (switchgrass and Bahiagrass), poplar cuttings, or was left unplanted.

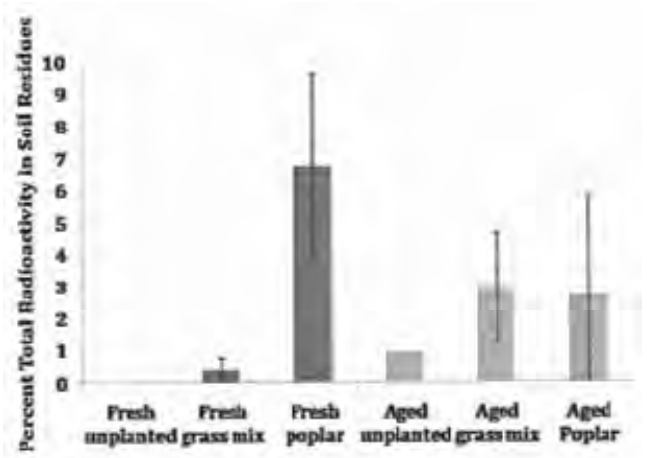


Figure 16. Percent of total radioactivity from [U-^{14}C]-TNT remaining in Lakeland soil residues following extraction by acetonitrile at the completion of the microcosm study. Soils were freshly contaminated with TNT at the beginning of the experiment (dark bars) or aged with TNT for 6 months (light bars) prior to the planting of a grass mixture (switchgrass and Bahiagrass), poplar cuttings, or was left unplanted.

Poplars in freshly contaminated Dorovan muck had the highest concentrations of TNT in both root and leaf tissues, at 13.3 and 6.5 mg/g, respectively (see Figure 17). Poplars in freshly contaminated Lakeland soil had the second highest concentrations of TNT with 1.4 and 2.5 mg/g in root and leaf tissues, respectively (see Figure 17). This is not a measure of total uptake throughout the microcosm study, but a measure of TNT remaining, unmetabolized in plant tissues. Concentrations of ADNTs in plant tissues were much higher, as shown in Figure 17. Plants in Lakeland soil had higher concentrations of ADNTs than plants in DM, with poplars having higher concentrations than grass. Poplars in fresh Lakeland soil had 44.8 mg/g ADNTs

in root tissues and poplars in aged Lakeland soil had 20.0 mg/g ADNTs in root tissues. ADNTs were predominantly found in root tissues rather than in leaf tissues. Grass in aged Lakeland soil also had substantial concentrations of ADNTs with 10.3 mg/g in root tissues. Radioactivity in plant tissues provides a look at TNT and metabolites, both extractable and unextractable. Figure 19 presents the percentages of total radioactivity, initially spiked in soils as [U-^{14}C]-TNT, in plant tissues. Plant tissues are divided into leaf, root, and stem for poplars only. Radioactivity was detected in all plant tissues, indicating all plants took up some TNT from soils and transported TNT from roots to other tissues. Poplars in aged Dorovan muck had 13% of the total radioactivity from their pots and poplars in fresh Dorovan muck and fresh Lakeland soil had 5% of their pots radioactivity. Grass in fresh Lakeland soil had 8% and grass in aged Dorovan muck had 5% of the total radioactivity, but other grass did not show much accumulation of TNT.

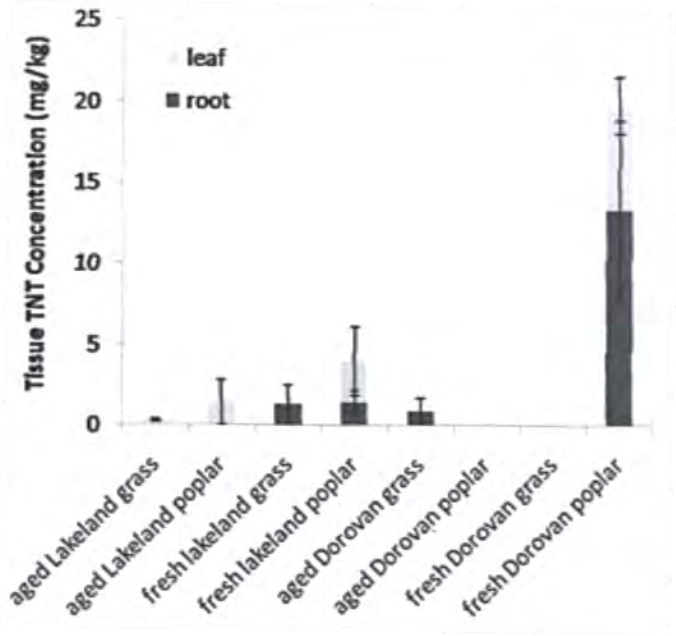


Figure 17. Concentration of extractable TNT from root and leaf tissues at the end of the microcosm study from pots planted with grass mixture (switchgrass or Bahiagrass) or poplar cutting. Stems were not analyzed. Error bars represent standard error from experimental triplicates.

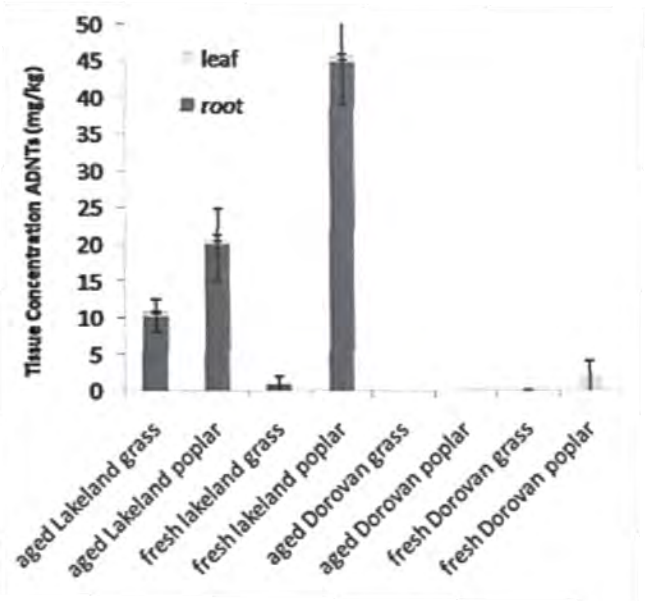


Figure 18. Concentration of extractable (by acetonitrile) ADNTs (2,4-ADNT and 4,6-ADNT), common metabolites of TNT, from root and leaf tissues at the end of the microcosm study from pots planted with a grass mixture (switchgrass and Bahiagrass) or poplar cuttings. Stems were not analyzed. Error bars represent standard error from experimental triplicates.

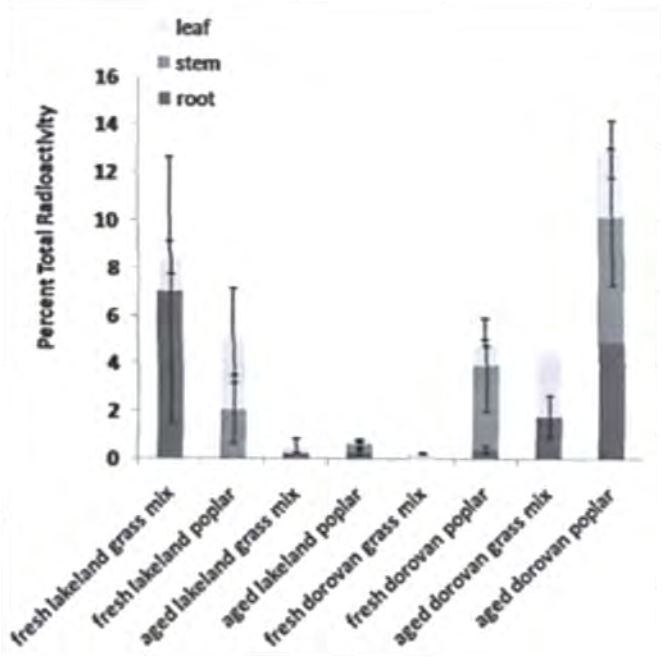


Figure 19. Percent total radioactivity, initially added to planter as [U-^{14}C]-TNT, residing in root, stem (poplars only), and leaf tissues planted in all soil types.

The physical-chemical characteristics of Dorovan muck and Lakeland soil, as shown in Table 3 and Table 4, did play important roles in determine the concentration of TNT in soils over time, both planted and unplanted. As expected, concentrations of TNT remained higher in Dorovan muck over time. Complete removal or transformation of TNT did occur in all freshly

contaminated soils. Aging played a significant role as well, TNT remained in aged Lakeland soil and Dorovan muck. The effects of aging were more pronounced in Dorovan muck. ADNTs concentrations in soils may point to microbial activities in soil. Dorovan muck pots had much higher concentrations of microorganisms as this soil generated over time from decaying plant matter and have a higher fraction of organic carbon to support microorganisms. Permanent binding of TNT to soil residues was not a large sink for TNT in the systems, accounting for >3% of total radioactivity in most systems. It is interesting to note that unextractable fractions of TNT were similar between aged and freshly contaminated soils, excepting the larger percentage identified in freshly contaminated Lakeland soil with poplars.

It is difficult to pick out a pattern of performance among the different plantings. Planting had little effect on the concentrations of TNT or ADNTs in soils over time. TNT was removed from soil just as effectively from unplanted soils. ADNTs concentrations were slightly higher in unplanted pots, which may explain some of the removal. Plants were taking up TNT, as demonstrated by extraction of TNT, ADNT, and radioactivity. The lack of efficiency in TNT removal in plants may be in part due to the difficulty of keeping plants alive during the study. Lakeland soil did not provide a good substrate for poplars and initial available concentrations of TNT in Lakeland soil may have been high due to the lack of sportive properties. Both soil types were fairly low in nutrients and soils were not amended with fertilizers in this study. In general, soil type and aging was a much better indicator TNT removal than the type of plant used. Microbial communities appear to play a significant role in the degradation of TNT in these pots.

Visualization of RDX and TNT Transport in Hybrid Poplar and Switchgrass using Phosphor Imager Autoradiography and Microscopy

Phosphor imager autoradiography is a technique for rapid, sensitive analysis of the fate of xenobiotics in plant tissues. Use of this technique is relatively new to research in the field of plant science, and the potential for enhancing visualization and understanding of plant uptake and transport of xenobiotics remains largely untapped. Phosphor imager autoradiography is used to investigate the uptake and translocation of the explosives TNT and RDX within *Populus deltoides nigra* DN34 (hybrid poplar) and *Panicum virgrat*um (switchgrass) Alamo.

Autoradiography has been used to investigate the fate of toxic xenobiotic compounds in plants (Woodard-Blankenship and Papin, 1995; Best et al., 1999; Feng, 2004; Hornik, 2005; Nepovim, 2005; Adamia et al., 2006; Chrikishvili, 2006; Cosio, 2006; Maracci, 2006; Soudek, 2006). These studies utilized x-ray, traditional, and emulsion film technologies that require lengthy exposure periods (20-60 days). Phosphor imager autoradiography is an emerging technique that allows for rapid, sensitive analysis of the fate of xenobiotics in plant tissues. The development of phosphor imager autoradiography opens up the possibilities of utilizing this technique for localization of ^{14}C-, ^{32}P-, ^{33}P-, or ^{35}S-labeled contaminants in plants. In addition, phosphor imager autoradiography has great potential to become a quick monitoring tool to assess the extent of radionuclide intake, such as 90Sr, by plants located around nuclear facilities. The most popular phosphor storage plate is composed of europium (Eu^{2+}) activated barium fluorobromide (BaFBr) phosphor which absorbs radiation emitted from a sample. The stored radiation energy is released as photons when the phosphor is scanned with visible light: the excitation wavelength of Eu:BaFBr photon is 633 nm and its emission wavelength is 390 nm. The energy of a photon is recorded and converted to a visible image (Schweizer, 2001). Major advantages of using phosphor storage plates for ^{14}C autoradiography are greater sensitivity, with 100 to 1000 times more sensitive than the film, and faster processing, requiring only one tenth of the time required compared to traditional film (Kanekal, 1995; Cole, 2003). Only a handful of

studies have begun to use phosphor imager technology to monitor uptake and transport of xenobiotics in plants (Sulmon, 2007; Vila, 2007).

The experiment explores the possibilities for visualization and quantification using phosphor imager autoradiography to assist investigation of the uptake and transport of the explosives RDX and TNT within root and leaf tissues of *Populus nigra* x *deltoids* DN34 (hybrid poplar) and *Panicum vigratum* (switchgrass) Alamo. Switchgrass performance in remediating TNT has been previously shown (Peterson et al., 1998; Dzantor et al., 2000; Chekol et al., 2002), but this is the first study to show uptake and translocation of toxic explosives in this plant.

Four week-old poplar plantlets, with similar transpiration rates, were exposed to a mixture of TNT and [U-^{14}C]-TNT (10 mg L^{-1} (44 μM), 70 nCi) or RDX and [U-^{14}C]-RDX (10 mg L^{-1} (45 μM), 70 nCi) for 48 h, after which the leaf and root sections were excised from the woody cutting for autoradiography analysis. Figure 20 shows the phosphor images 48 h after spiking hydroponic solutions with ^{14}C-RDX mixture or [U-^{14}C]-TNT mixture. The rapid translocation of ^{14}C-RDX and ^{14}C-metabolites in poplars is evident, with 90.9 (± 1.8)% of the label identified in leaf tissues, 4.4 (± 1.6)% in the stem, and 3.9 (± 0.3)% in root tissues, based on signal intensity in the image. The lack of translocation of [U-^{14}C]-TNT and metabolites is also apparent in the image of the poplar plantlet exposed to [U-^{14}C]-TNT, in which label is only detected in root tissues. Aliquots of the hydroponic media taken from 48 hrs yielded no image, indicating that the activity was removed below the detection limits of the phosphor imager.

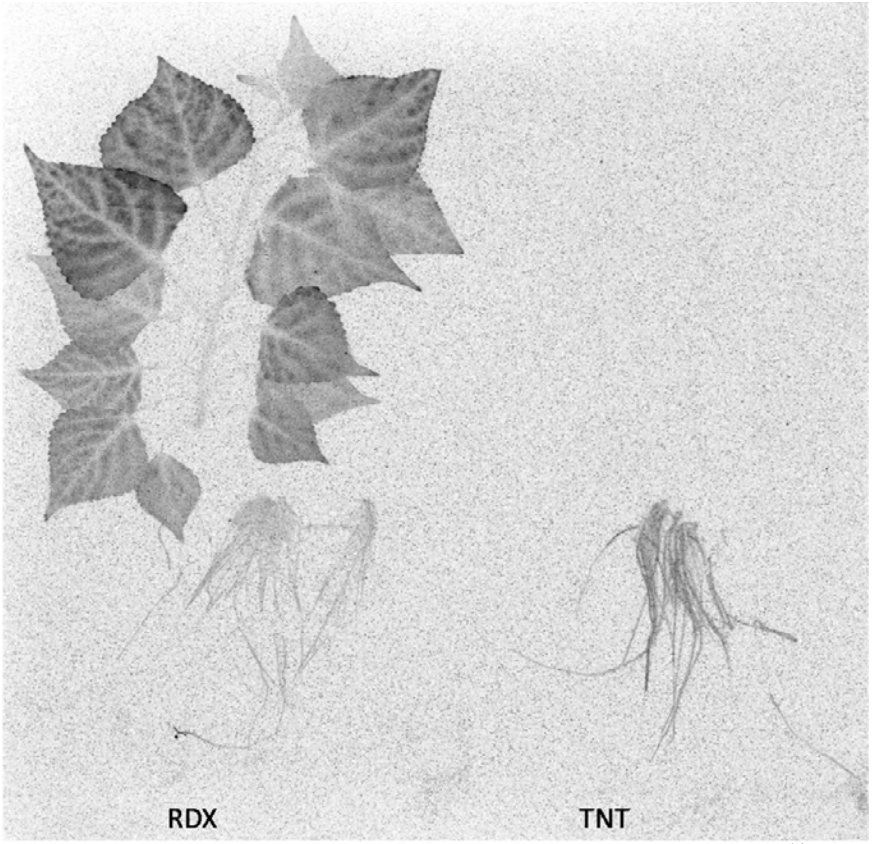


Figure 20. Phosphor imager autoradiograph shows the distribution of [U-^{14}C]-TNT or -RDX by poplars 48 h after exposure. Leaf sections and root sections were excised prior to drying and imaging.

RDX translocation was further investigated in poplars by exposing two four week-old poplar plants with similar transpiration rates to the RDX and ^{14}C-RDX mixture (40 mg L^{-1} (0.18 mM)) for 8 h (7 uCi) and 120 h (70 uCi). Higher radioactivity for the120 h-exposed plant was needed to allow detection of ^{14}C signals at a microscopic level. Fast uptake and translocation of ^{14}C-RDX or ^{14}C-metabolites to poplar leaf tissues was apparent after 8 h (Figure 21A). Figure 21A shows an asymmetrical distribution pattern of ^{14}C labels after 8 h of exposure. 120 h after exposure, ^{14}C labels were spread to all leaves (Figure 21B), with 59.5% of the radiolabel taken up by plants recovered in leaf tissues. As shown by darker points, higher concentration of ^{14}C labels was distributed to the edge of leaves and older leaves. The radiolabel in these leaf tissues is much more discrete compared to younger leaves (Figure 21B). Four leaf samples were collected from a similar height at 24, 48, 96 and 120 h after exposure. Quantitative analyses using a bio-oxidizer confirmed that radioactivity concentration in leaves increased with time up to 120 h. Microscope-level autoradiographs of the leaf sections collected after 120h exposure to ^{14}C-RDX are shown in Figure 22. Stronger ^{14}C activity was detected specifically around chloroplasts and lignified tissues (Figure 22B and C), indicating translocation of RDX or its metabolites into chloroplasts or incorporation of these molecules into plant structure (i.e., cell wall).

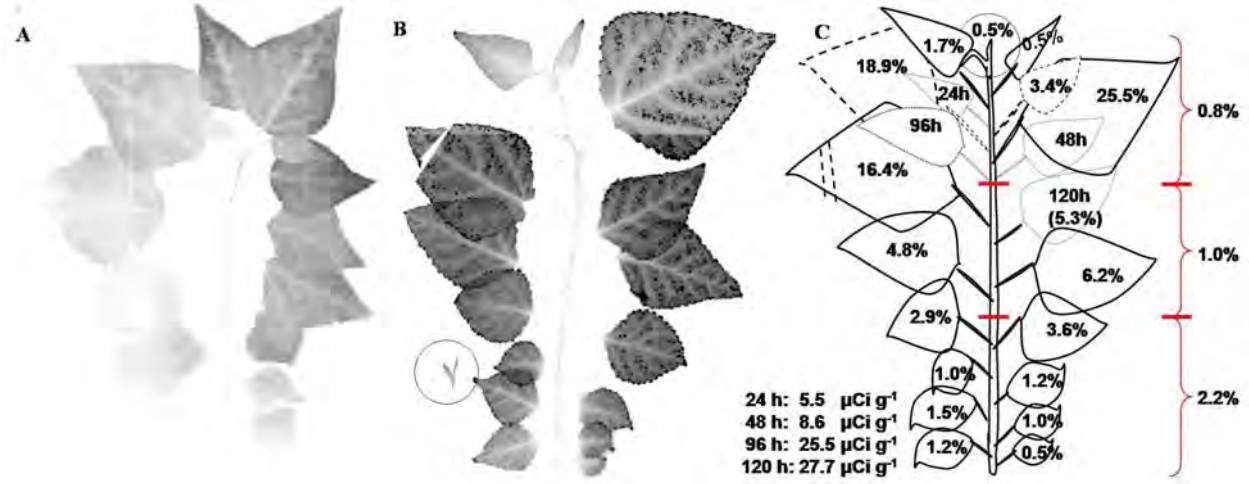


Figure 21. Phosphor imager autoradiographs show the activity from ^{14}C-RDX and metabolites in poplars after 8h exposure (7 µCi) (A) and after 120 h exposure (70 µCi) (B). A leaf samples was excised before phosphor autoradipgraphy image was captured, as indicated by dashed lines, to for microautoradiography analysis. A diagram to show distribution of ^{14}C activity within leaf tissues and stem sections after 120 h is provided (C). Leaf samples taken after 24, 48, 96 and 120 h from a similar height were analyzed by bio-oxidation and liquid scintillation counting to track accumulation of activity in leaves, based on dry weight, over time (C).

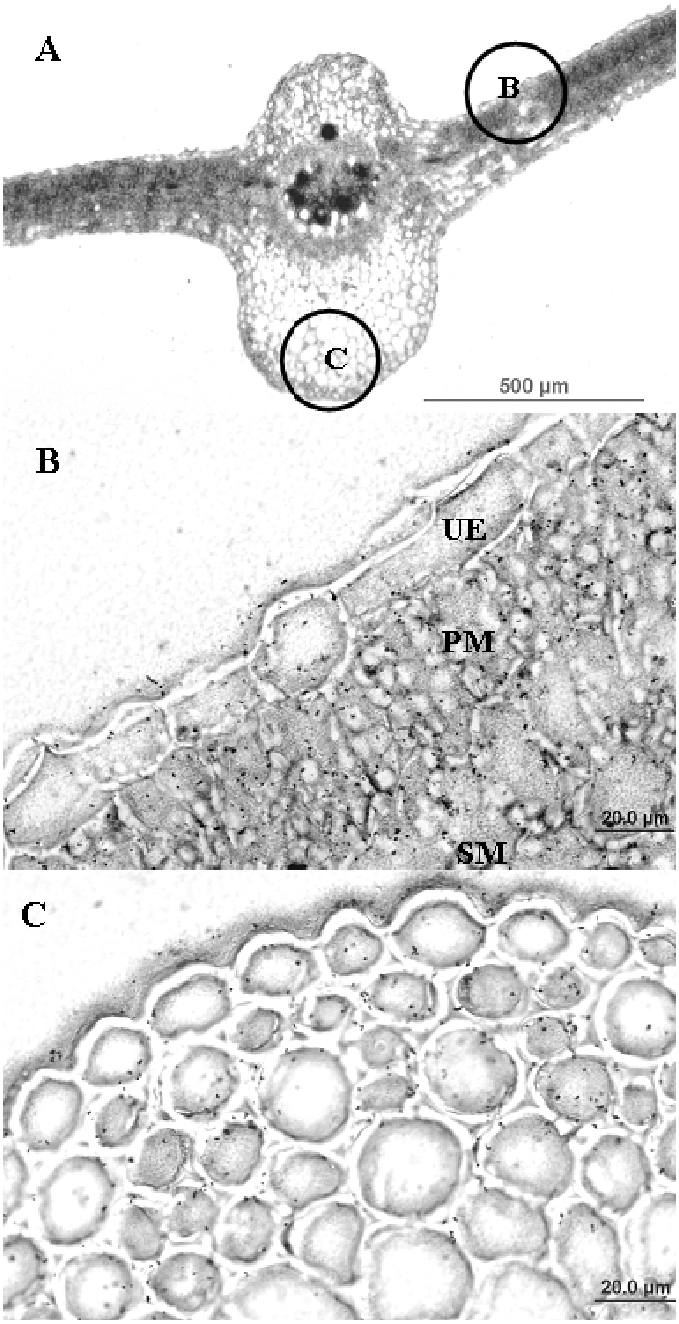


Figure 22. Microautoradiographs of the leaf sections excised from poplar exposed to 14C-RDX, shown in Fig.2B, after 96h. Images are of a leaf-cross section (A). A microautoradiograph shows 14C signals from 14C-RDX or its metabolites (B) (UE: upper epidermis cell, PM: palisade mesophyll cell, SM: spongy mesophyll cell). A third microautoradiograph shows 14C-signals association with lignified cells (C). Radioactivity appears as dark specks in the images.

Figure 23 is an image of a time course experiment of four week-old switchgrass plants spiked with 10 mg L^{-1} TNT (22 µM), 70 nCi [U-^{14}C]-TNT sequentially 120, 72, 48, 24 and 8 h before being sacrificed at the same time. The plants were set up on a single piece of chromatography paper to undergo drying, exposure to phosphor screen, and imaging simultaneously. TNT remains predominantly (>95%) in root tissues throughout the time course. However, after only 8 h, some translocation of the [U-^{14}C]-TNT and [U-^{14}C]-TNT-metabolites from root to leaf tissues is faintly visible. Slightly more becomes apparent after 24 and 48 h, but little changes in the intensity of the radiolabel in leaf tissues of switchgrass after 48 h.

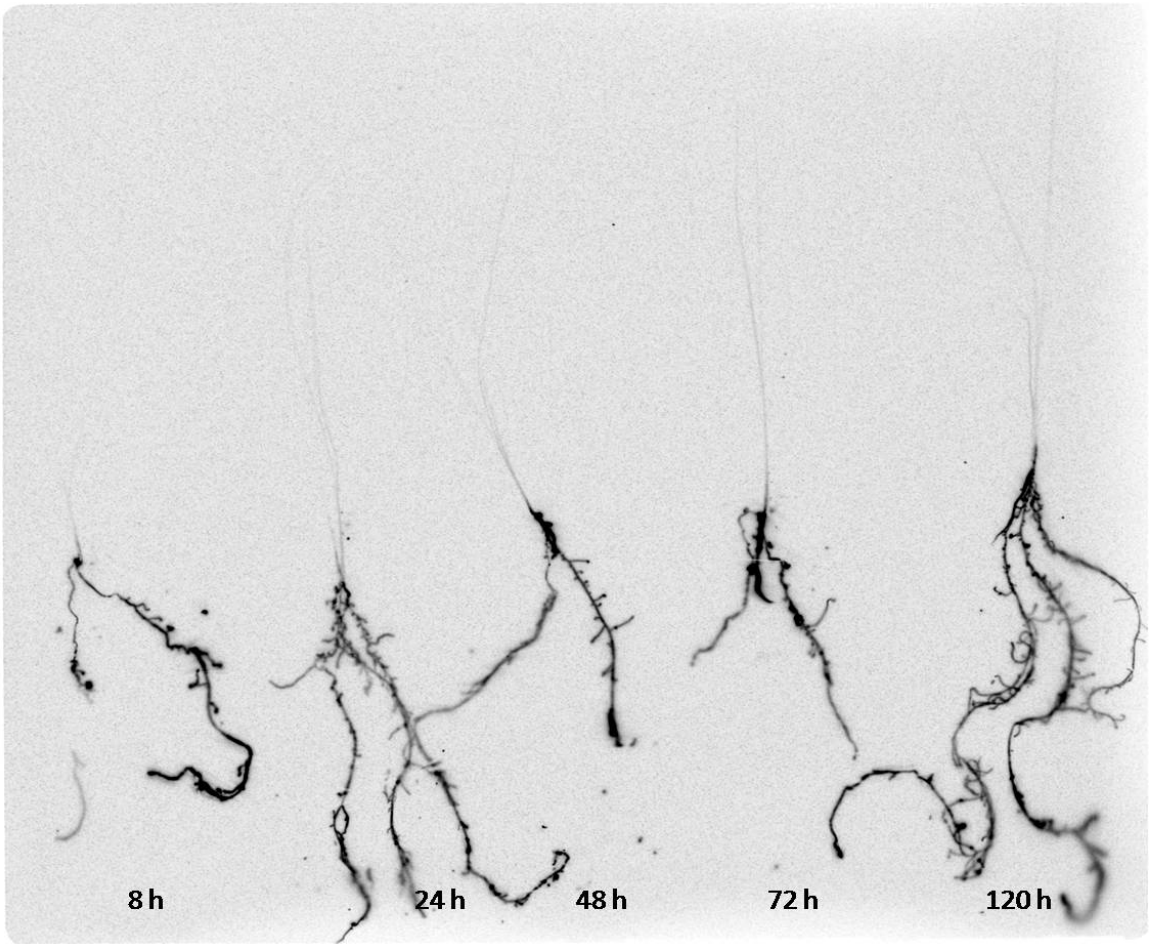


Figure 23. Phosphor imager autoradiograph showing distribution of [U-^{14}C]-TNT after 8, 24, 48, 72, and 120 h. Switchgrass plants are in-tact and were dried and imaged simultaneously.

Six week-old switchgrass plants, with similar transpiration rates, were exposed to TNT or RDX cold and radiolabeled mixtures (10 mg L^{-1}, 70 nCi). Figure 24 and Figure 25 show the phosphor image autoradiographs of switchgrass 48 h after exposure to the RDX or TNT mixture. As in poplar, RDX and its metabolites are readily translocated to leaf tissues (Figure 24). However, the RDX is much more evenly distributed between leaf and root in switchgrass compared to poplar. Of the radiolabel taken up by the switchgrass plant, 41.9 (±0.2)% was found in roots while 58.1 (±0.2)% was found in leaf tissues. As in the poplar, TNT remained predominantly in the roots, although the leaves are faintly visible, indicating the detection of radioactivity. Again, aliquots of the hydroponic media were included but were not detected in either image, indicating complete removal of the radiolabel from solution.

Figure 24. Phosphor imager autoradiograph of distribution of [U-^{14}C]-RDX in switchgrass 48 h after exposure.

Figure 25 presents an example of semi-quantitative analysis of radioactivity using phosphor imager autoradiography with a calibration. Aliquots of known radioactivity were used to create a calibration. The images of the calibration aliquots are shown in the top left corner of Figure 25. Table 5 presents the radioactivity in the roots and leaves of the switchgrass plants as calculated from the calibration curve. Of the radiolabeled TNT taken up by the switchgrass, 97.5 (±0.10)% remained in the roots while 2.5 (±0.15)% was transported to leaf tissues (of the initial radioactivity, 96.4 (±0.10)% was detected in roots and 2.4 (±0.15)% was in leaf tissues).

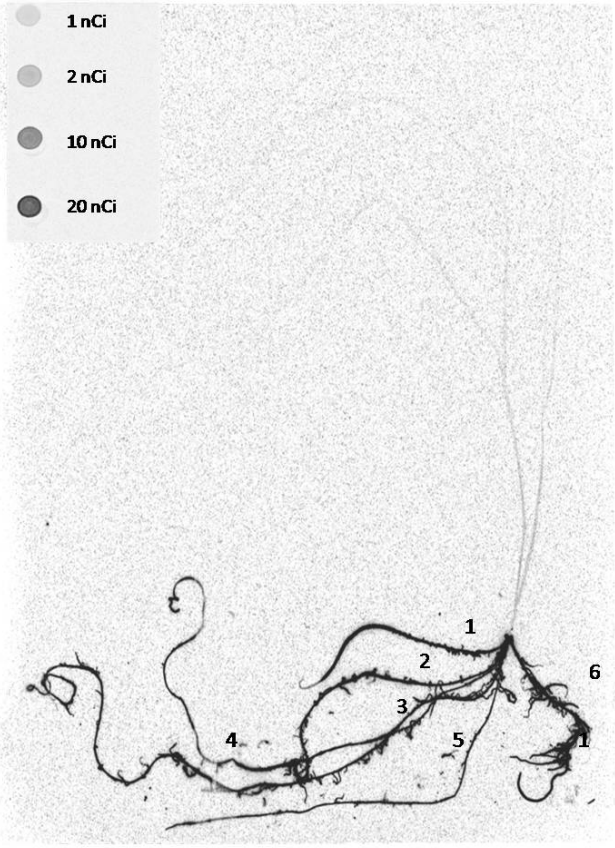


Figure 25. Phosphor imager autoradiograph of switchgrass 48 h after exposure to [U-^{14}C]-TNT. The upper left corner shows the aliquots of [U-^{14}C]-TNT of known radioactivity used as calibrators to calculate distribution of radioactivity in the plant. Numbered roots correspond to values given in Table 5.

Table 5. Calculated radioactivities in root and leaf sections of switchgrass 48 h after exposure to [U-^{14}C]-TNT, as shown in Figure 24.

	Activity (nCi)	Standard error	% Total Activity
root 1	8.68	0.11	12.39
root 2	9.06	0.19	12.95
root 3	17.47	0.72	24.96
root 4	10.86	0.08	15.52
root 5	6.05	0.20	8.64
root 6	15.36	0.34	21.95
leaf	1.70	0.10	2.43
total root	67.49	0.07	96.41
total activity	69.18	0.06	98.83
unaccounted	0.82	0.06	1.17

Phosphor imager autoradiography is easily applicable to research in plant science, and phytoremediation in particular, as it greatly enhances the observation of xenobiotics within plants. It provides an easy, visual comparison between contaminants and between plant types. This paper presents the use of phosphor imager autoradiography of phytoremediation of explosives contaminants by poplars and switchgrass. TNT is bound quite strongly in root tissues while RDX is readily translocated to leaf tissues in a short amount of time. After 48 h, RDX and RDX-metabolites are more evenly distributed between roots and leaves in switchgrass compared to poplars, although this may be attributed to the more even ratio of root: leaf tissue in switchgrass compared to poplar. Transport of TNT and TNT-metabolites to leaf tissues is limited in switchgrass, but transport is visible to leaf tissues of switchgrass after as little as 24 h. [U-^{14}C]-TNT radiolabel was not visible in poplar leaf tissues after the same exposure. Phosphor imager autoradiography provides a striking visual component to these observations.

While the fate of the explosives in poplar tissues was anticipated based on previous research (Thompson et al., 1998; 1999), autoradiography allows for more specific localization of xenobiotic fate to specific leaf types and observation of potential compartmentalization of RDX and RDX-metabolites within poplar leaf tissues. The asymmetrical pattern observed 8 h after exposure to RDX is another interesting observation greatly aided by autoradiography. Cosio et al. (2005) reported similar distribution pattern of cadmium (^{109}Cd) in *Thlaspi caerulescens* using an autoradiography X-ray film. The autoradiographs highlight the importance in understanding the uptake patterns when selecting tissues for analysis, to determine chemical concentration, enzyme induction levels or other investigations important for phytoremediation research.

The ease of producing autoradiographs using a phosphor imager should encourage further use of this technique to complement studies in phytoremediation. Because phosphor imager autoradiography produces a digital image, over or under exposure is not an issue. Phosphor imager autoradiography does not require a dark room and the phosphor storage plate is reusable (Schweizer, 2001; Cole, 2003). The storage plate can be quickly erased and the sample reexposed, particularly when working with radiolabels with a long half-life such as ^{14}C. Because plants are not chemically treated in the process it is possible to use phosphor imager autoradiography in conjunction with other analytical techniques, including analysis of radioactivity using bio-oxidation and liquid scintillation counting.

This is the first report of using autoradiography for the quantification of uptake and translocation of a xenobiotic in plants. Phosphor imager autoradiography makes this possible by producing a digital image in which activity can be interpreted in the intensity of the gray value of the pixels of the image. The semi-quantitative autoradiography provides an alternative or complement to analytical mass balance approaches to investigations of plant uptake. This technique could be further expanded to determine activities in plant tissues and environmental samples with unknown initial activities. Other possible applications of phosphor imager autoradiography include assessing exposure levels of natural radiation such as radon, uranium or other radioactive decay products in abiotic or living matter (Cole, 2003) and understanding the fate of toxic environmental pollutants in ecosystems. Due to the greater sensitivity of phosphor imager autoradiography, doses of radioactive compounds used in experiments and required exposure periods are considerably less than those of traditional film autoradiography. The possibilities for exploring phytoremediation using phosphor imager autoradiography are seemingly endless.

The transformation and uptake of perchlorate in hydroponic solution and soil sludge systems (Lakeland soil and Dorovan muck) was investigated through 70-day greenhouse experiments using two plants: hybrid poplar and switchgrass. For unplanted systems, the concentration of perchlorate was reduced in hydroponic solution, sandy soil sludge, and muck soil sludge by 5.8%, 10.8%, and 17.9%, respectively. This indicates the perchlorate could be transformed by bacterial strains, but slowly. The presence of the tested plants highly enhanced the dissipation of perchlorate dependent on plant species and soil types. For poplar tree, perchlorate dissipations in hydroponic solution, sandy soil sludge, and muck soil sludge were 31.5%, 69.2% and 50.0%, respectively. For switchgrass, the respective dissipation for the three systems was 25.5%, 32.8% and 57.7%. Interestingly, the presence of soil obviously promoted the performance of plants. The recorded transpiration of hybrid poplar was much larger than that of switchgrass in exposed experiments, but the performance of switchgrass was much better than that of hybrid poplar in the reduction of perchlorate. In addition to plant-uptake, these observations suggest that biodegradation in rhizosphere significantly contributed to the abatement of perchlorate pollution.

Plant Uptake of Explosives in Soil-Slurry Systems

Poplar plantlets were exposed to 10 mg/L (44 µM) [U-^{14}C]-TNT in batch hydroponic and soil slurry systems to examine uptake of TNT and TNT metabolites from solutions. Radioactivity and TNT concentrations remained fairly constant in unplanted hydroponic controls for 14 days of the experiment. But the TNT concentration decreased rapidly in the planted system and was diminished in only two days, while the radio-labeled concentration which included both metabolites and parent-TNT decreased more slowly indicating the presence of intermediate metabolites (see Figure 26).

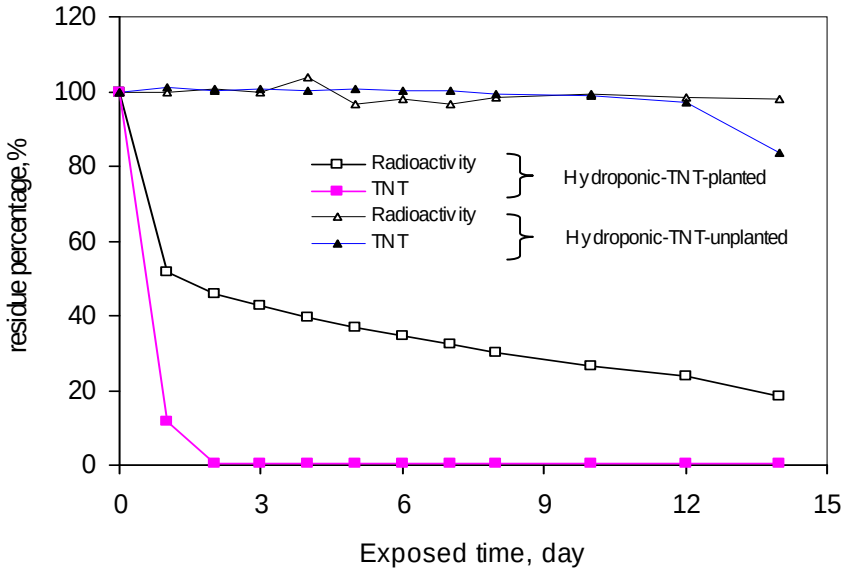


Figure 26. Dissipation percentage of radioactivity and TNT parent compound in solution when poplar plantlets were exposed to 10 mg/L of [U-14C] TNT in hydroponics.

In unplanted Dorovan muck soil slurries, both TNT and radioactivity concentrations decreased at the same rate, presumably due to sorption of TNT by soil particles and the lack of bioavailability for metabolism by root tissues (see Figure 27). However, in unplanted Lakeland (sandy) soil slurries the parent compound decreased more rapidly than total radioactivity, suggesting microbial and/or plant degradation was occurring in these reactors – TNT was more bioavailable in the sandy soil for biotransformation (see Figure 27). Parent compound TNT concentrations decreased below detection after 48 hrs in all reactors. Radioactivity was more gradually removed from planted systems, demonstrating poplar uptake of TNT metabolites.

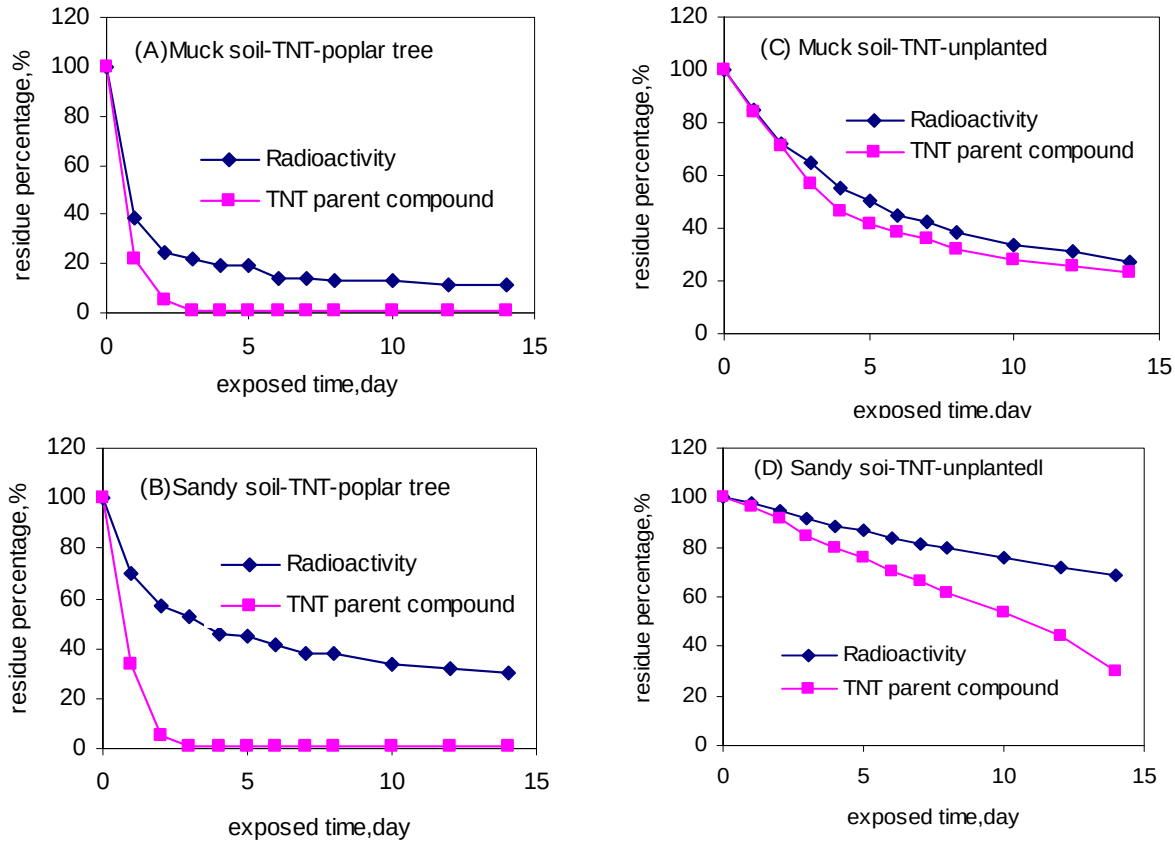


Figure 27. Dissipation percentage of radioactivity and TNT parent compound in solution when poplar plantlets were planted or unplanted to 10 mg/L of [U-^{14}C] TNT in muck soil and sandy soil.

Distribution of radioactivity within the systems is shown in Figure 28. In hydroponic systems with plants, 70% of the TNT was taken up by the poplar plant, followed by 38% taken up in the sandy soil and only 27% in the muck soil. Clearly, bioavailability is strongly affected by the strength of sorption to soils (log K_{oc}). In soil-slurry systems, much of the TNT was in soil residues, with the greatest percentage residing in Dorovan muck slurries. Plant uptake also played an important role in planted reactors, with the greatest uptake occurring in the hydroponic system.

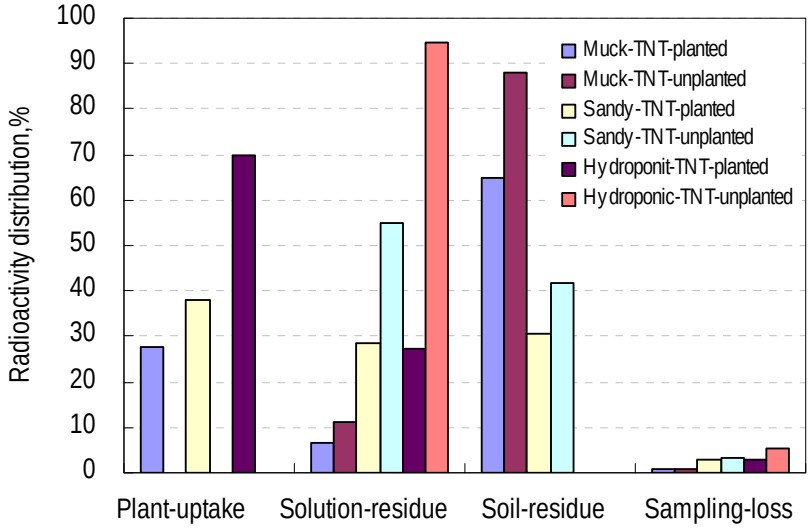


Figure 28. Distribution of radioactivity in the soil slurry-poplar tree system where poplar plantlets were exposed to 10 mg/L of [U-^{14}C] TNT for 14 days.

Figure 29 shows the distribution of [U-^{14}C]-RDX in hydroponic and soil-slurry systems. Sorption to soil is much less important in Lakeland (sandy) soil systems. Plant uptake was significant in all planted systems, with greatest plant uptake occurring in hydroponic systems followed by Lakeland soil slurries. Dorovan muck sorption interfered with plant uptake to a large extent.

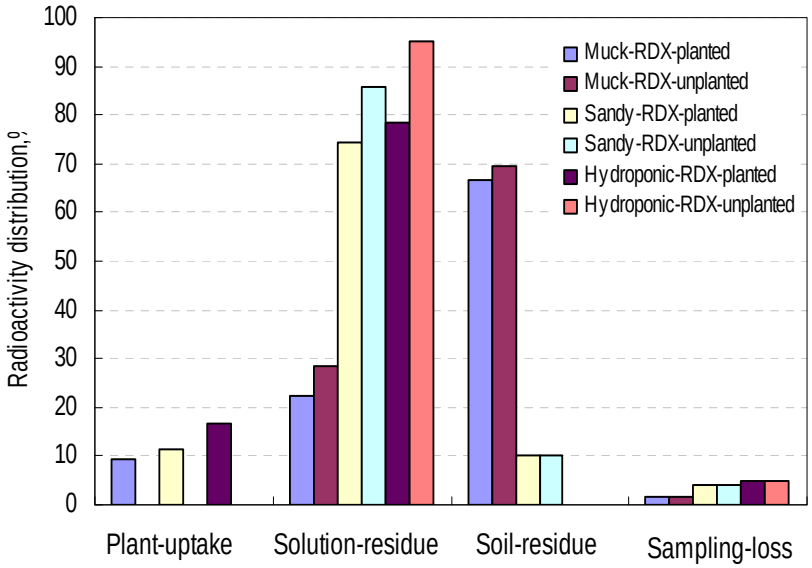


Figure 29. Distribution of radioactivity in the soil slurry-poplar tree system where poplar plantlets were exposed to 10 mg/L of [U-^{14}C] RDX for 14 days.

Biodegradation of TNT, RDX, and HMX in Unplanted Soils from Eglin AFB

To better understand and differentiate the removal mechanisms for the energetic compounds TNT, RDX, and HMX, a variety of microcosm studies have been performed using soils taken from Eglin AFB in Okaloosa County, Florida. Examples in the literature show degradation and removal of TNT, RDX, and HMX from soil by both bacteria and plants (Hannink, et al., 2002; Hawari, et al., 2000). This indicates that both microbes and plants are likely to play important roles in any phytoremediation application. Microcosm studies using unplanted soil provide an estimate for background degradation rates for comparison to phytoremediation removal rates.

Soil used in the experiment was unplanted and either contaminated immediately prior to incubation or was contaminated and aged for 18 months while kept at 4 °C. The experimental set up for freshly contaminated soil consisted of soil which was: sterile and kept in the light, sterile and kept in the dark, non-sterile and kept in the light, or non-sterile and kept in the dark. The experimental setup for soil which was contaminated and aged for 18 months consisted of soil which was non-sterile and either kept in the light or dark. The experimental setup is provided in Table 6.

Table 6. Experimental setup for unplanted soils experiment using Lakeland Soil and Dorovan muck.

	Freshly Contaminated	Aged 18 Months
Sterile, Light	TNT, RDX, HMX	-
Non-Sterile, Light	TNT, RDX, HMX	TNT, RDX
Sterile, Dark	TNT, RDX, HMX	-
Non-Sterile, Dark	TNT, RDX, HMX	TNT, RDX

As shown in Figure 30, Figure 31, and Figure 32, soil samples taken over the course of 56 days showed no statistically significant degradation of RDX or HMX in any of the soil microcosms. Minimal degradation of RDX and HMX could have occurred but may have been masked by the error associated with the variability in sampling each pot. However, as the experiment took place over 56 days, any minimal degradation would still indicate very slow removal kinetics for RDX and HMX.

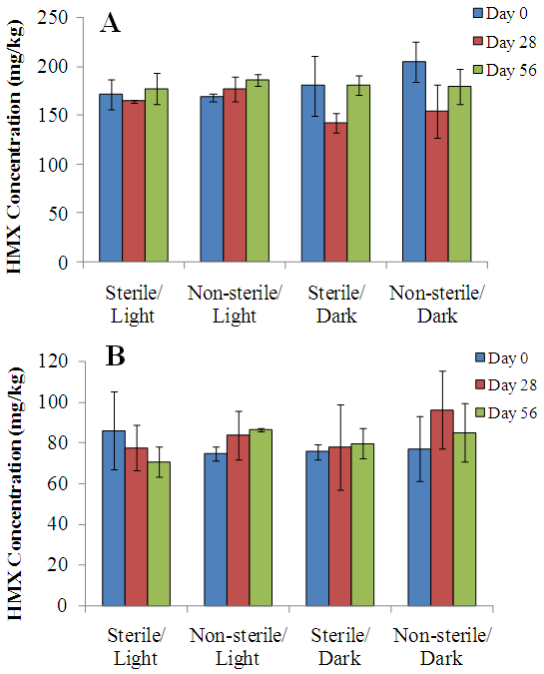


Figure 30. Degradation experiment of HMX incubated in (A) Dorovan muck and (B) Lakeland Soil. Error bars represent one standard deviation.

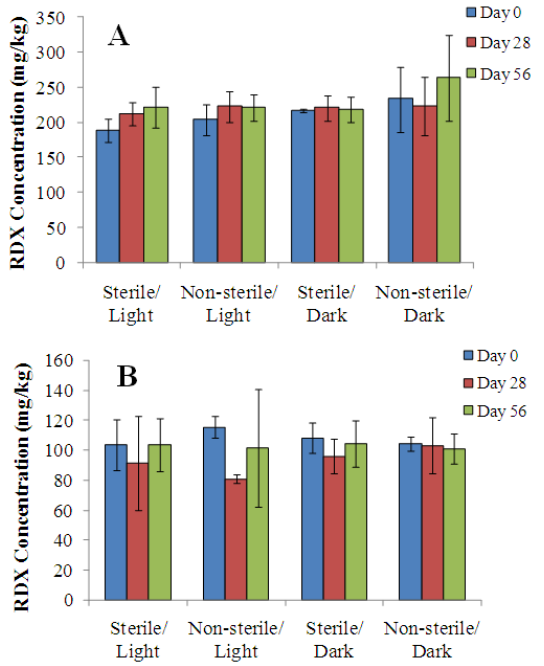


Figure 31. Degradation experiment of RDX incubated in (A) freshly contaminated Dorovan muck, (B) freshly contaminated Lakeland Soil.

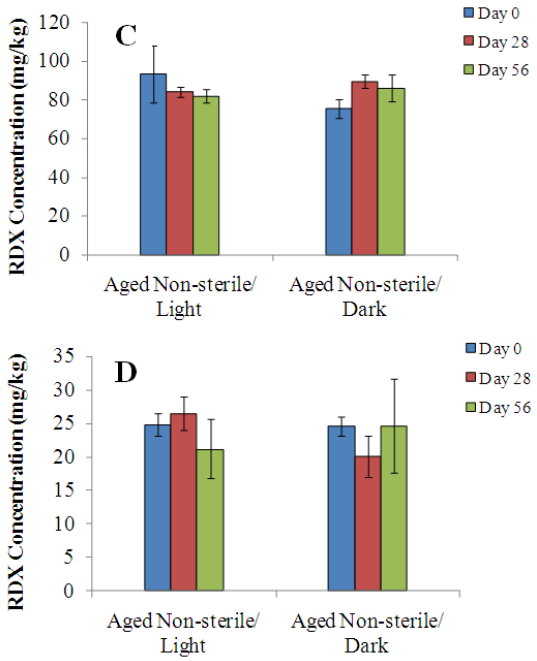


Figure 32. Degradation experiment of RDX incubated in (C) aged Dorovan muck, (D) aged Lakeland Soil.

TNT degraded rapidly in microcosms which were freshly contaminated as shown in Figure 33. In freshly contaminated Dorovan muck, greater than 50% removal was achieved within the first 7 days with the exception of the sterile soil which was kept in the light. Non-sterile Dorovan muck kept in the light and dark achieved 90% and 91% removal, respectively, over 56 days. Less degradation occurred in sterile Dorovan muck, which achieved 40% removal in the light and 89% removal in the dark over 56 days.

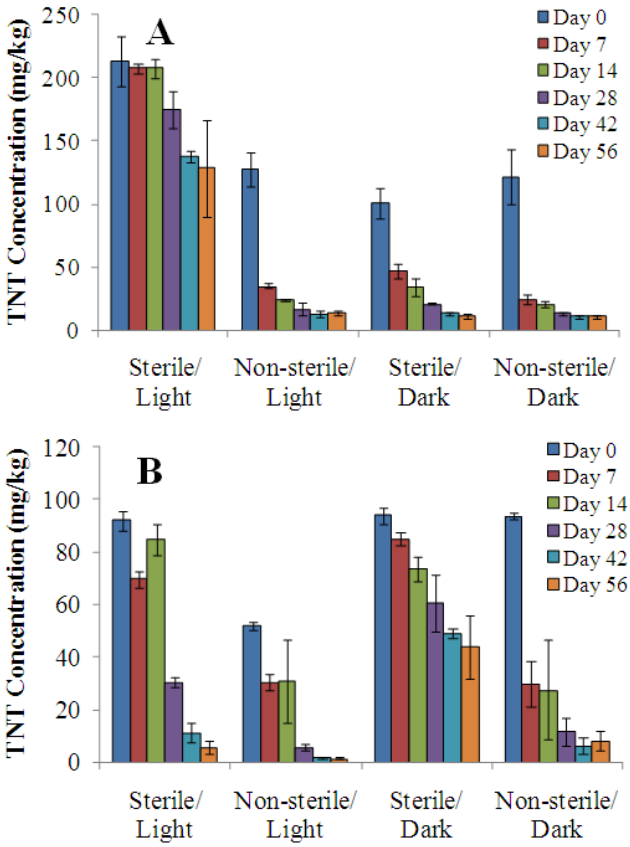


Figure 33. Degradation experiment of TNT incubated in (A) freshly contaminated Dorovan muck and (B) freshly contaminated Lakeland Soil. Error bars represent one standard deviation.

Lakeland Soil which was freshly contaminated with TNT showed similar results to Dorovan muck. Rapid degradation occurred in non-sterile, freshly contaminated Lakeland Soil with 89% and 88% removal in the light and dark, respectively, over the first 28 days. Over 56 days, the non-sterile freshly contaminated Lakeland Soil achieved 97% removal in the light and 93% removal in the dark. Degradation in sterile, freshly contaminated Lakeland Soil occurred more slowly than in non-sterile Lakeland Soil. TNT removal of 94% in the light and 53% in the dark occurred in sterile, freshly contaminated Lakeland Soil.

Degradation of TNT in aged Dorovan muck was much lower compared to aged Lakeland Soil over the 56 day experiment as shown in Figure 34, likely due to increased binding in the higher clay content of Dorovan muck. TNT removal in aged Dorovan muck was 19% in the light and 36% in the dark. Aged Lakeland Soil achieved 97% and 93% removal in the light and dark, respectively.

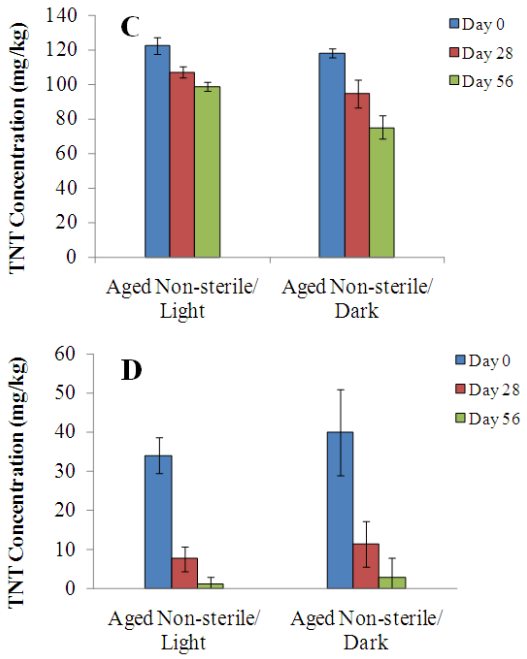


Figure 34. Degradation experiment of TNT incubated in (C) aged Dorovan muck and (D) aged Lakeland Soil. Error bars represent one standard deviation.

The target concentration of 100 mg/kg for TNT was not obtained in all of the experimental setups shown in Figure 33. This could be due to particles of undissolved TNT that may have been present in the stock solution used to contaminate the soils. Different starting concentrations, especially the higher TNT concentration present in the sterile Dorovan muck in the light, could have made an impact on removal rates throughout the experiment.

The majority of metabolites detected during the TNT microcosm study were 2-ADNT (2-amino-4,6-dinitrotoluene) and 4-ADNT (4-amino-2,6-dinitrotoluene). Trace amounts of 2,4-DNT (2,4-diamino-6-nitrotoluene) and 2,6 DNT (2,6-diamino-4-nitrotoluene) were also detected at concentrations much lower than those of 2-ADNT and 4-ADNT. As shown in Figure 35 and Figure 36, the total ADNT concentrations are much higher in Dorovan muck than in Lakeland Soil. ADNT concentrations increased rapidly (within 7 days) in freshly contaminated Dorovan muck, with the exception of sterile soil kept in the light. Minimal ADNT concentrations were present in freshly contaminated Lakeland Soil over the 56 day experiment. As with freshly contaminated soil, aged Dorovan muck contained higher levels of ADNTs than aged Lakeland Soil.

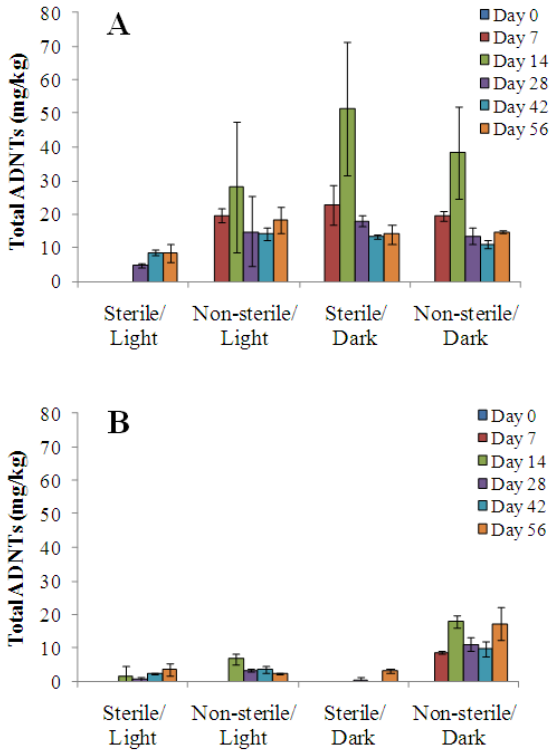


Figure 35. TNT metabolites 2-ADNT and 4-ADNT measured in (A) freshly contaminated Dorovan muck and (B) freshly contaminated Lakeland Soil. Error bars represent one standard deviation.

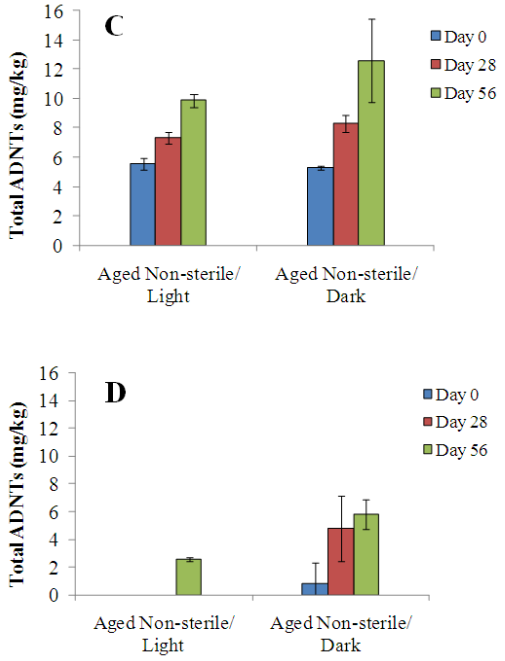


Figure 36. TNT metabolites 2-ADNT and 4-ADNT measured in (C) aged Dorovan muck and (D) aged Lakeland Soil. Error bars represent one standard deviation.

The results of the degradation experiments provided in Figure 30, Figure 31, and Figure 32 show that HMX and RDX are recalcitrant under moist, aerobic conditions in the two soils which were investigated. These findings are consistent with other results in the literature showing the relative stability of RDX and HMX under aerobic conditions (Grant, Jenkins, Myers, & McCormick, 1995; Thomas F. Jenkins, et al., 2003; McCormick, Cornell, & Kaplan, 1981). The majority of explosives contamination at military testing ranges is primarily present in the top 5 cm of the ground surface (generally aerobic), which may explain the recalcitrance of these compounds under actual field conditions (Alan D. Hewitt, et al., 2007).

In contrast to RDX and HMX, TNT disappeared relatively quickly under most experimental conditions. All microcosms with freshly contaminated soil showed significant removal of TNT, regardless of soil type or being sterile. As shown in Figure 33, TNT degraded most consistently in non-sterile soil, with approximately 90% removal for both soil types in the light and in the dark. More variable results occurred among sterile soils. TNT removal in sterile soil ranged from 40% (Dorovan muck, light) to 94% (Lakeland Soil, light). Although degradation occurred more slowly in the sterile soils, significant removal did occur over the 56 day experiment. This may indicate irreversible binding to clay, an abiotic degradation mechanism for TNT, or bacterial contamination into the soil microcosms over the course of the experiment.

Results from the aged TNT microcosms showed significant differences between removal rates in the two soil types, as shown in Figure 34. Much lower removal was observed in aged Dorovan muck compared to both aged Lakeland Soil and freshly contaminated soil of both types. Removal of only 19% (light) and 36% (dark) occurred in aged Dorovan muck. The substantially greater TNT removal of 97% (light) and 93% (dark) in aged Lakeland Soil is likely due to the much higher clay content present in Dorovan muck. TNT has been shown to adsorb to clay minerals, which may cause a decrease of TNT bioavailability to bacteria in the process (Haderlein, Weissmahr, & Schwarzenbach, 1996).

As expected, 2-ADNT and 4-ADNT were the dominant metabolites formed from TNT degradation throughout the experiment. As shown in Figure 35, higher concentrations of ADNTs remained in Dorovan muck than in Lakeland Soil. This is likely due to decreased bioavailability in Dorovan muck causing degradation to occur more slowly than in Lakeland Soil. Similar results were observed for ADNT concentrations in aged soils, as shown in Figure 36. As in freshly contaminated soil, higher concentrations of ADNTs were present in aged Dorovan muck than in aged Lakeland Soil throughout the experiment.

In conclusion, this experiment has shown that RDX and HMX are recalcitrant under aerobic conditions in both soil types that are present at Eglin AFB. These are expected results and are similar to the findings observed in the literature regarding the general recalcitrance of these compounds under aerobic conditions (Grant, et al., 1995; Thomas F. Jenkins, et al., 2003; McCormick, et al., 1981). In contrast, TNT was shown to disappear relatively quickly with some degradation to 2-ADNT and 4-ADNT, as well as trace amounts of 2,4-DNT and 2,6-DNT. This pathway for bacterial degradation of TNT under aerobic conditions has been well documented and is now confirmed to occur in Eglin AFB soils (Esteve-Nunez, Caballero, & Ramos, 2001).

Phytoremediation of RDX in Soil from Eglin AFB Planted with Bahiagrass and Hybrid Poplar

In order to ensure the validity of applying phytoremediation for the treatment of RDX contamination at Eglin AFB, a microcosm study was performed using the same plant species and soil utilized in the field study. The laboratory study consisted of five microcosms using native Lakeland soil from Eglin AFB planted with either Bahiagrass Pensacola (*Paspalum notatum*), the excised roots of Bahiagrass Pensacola, hybrid poplar (*Populus deltoides x nigra,* DN34), the excised roots of poplar, or remained unplanted as a control. The study included triplicates of each microcosm. Figure 37 shows the experimental setup of the Bahiagrass Pensacola, excised roots of the grass, and the control. The experiment was conducted for 56 days with soil samplings at days 0, 14, 28, and 56. On day 56, the plants were sacrificed. The leaves and roots of the poplars and the blades and roots of the Bahiagrass were excised for analysis. The roots excised at the start of the experiment were extracted and analyzed. All roots were rinsed thoroughly of soil prior to processing.

Figure 37. The experimental setup for the RDX microcosm study. Includes three replicates planted in soil from Eglin AFB with Bahiagrass Pensacola, three with excised Bahiagrass Pensacola roots, and three unplanted. All replicates were covered with aluminum foil to prevent the growth of phototrophic organisms. Not shown: the poplar and excised poplar root microcosms.

The study on the hybrid poplar microcosms was ended prematurely on day 40 due to a spider mite (*Tetranychus urticae*) infestation in the lab which caused the health of the poplars to decline rapidly. The other three microcosm studies were carried out for the entire 56 day period. As shown in Figure 38, substantial root density was achieved with the Bahiagrass. The results of the study are shown in Figure 39. The RDX concentration in soil was reduced by 77.7% after 14 days and 98.6% after 56 days in the planted Bahiagrass microcosm. The RDX concentration in soil was reduced by 96.3% after 14 days and 99.1% after 40 days in the planted poplar microcosm. The excised roots and unplanted control saw no significant reduction in

concentration over the 56 day period. There were no detectable concentrations of RDX found in the excised leaves, blades, or roots.

Figure 38. Significant Bahiagrass Pensacola root density was achieved by completion of study.

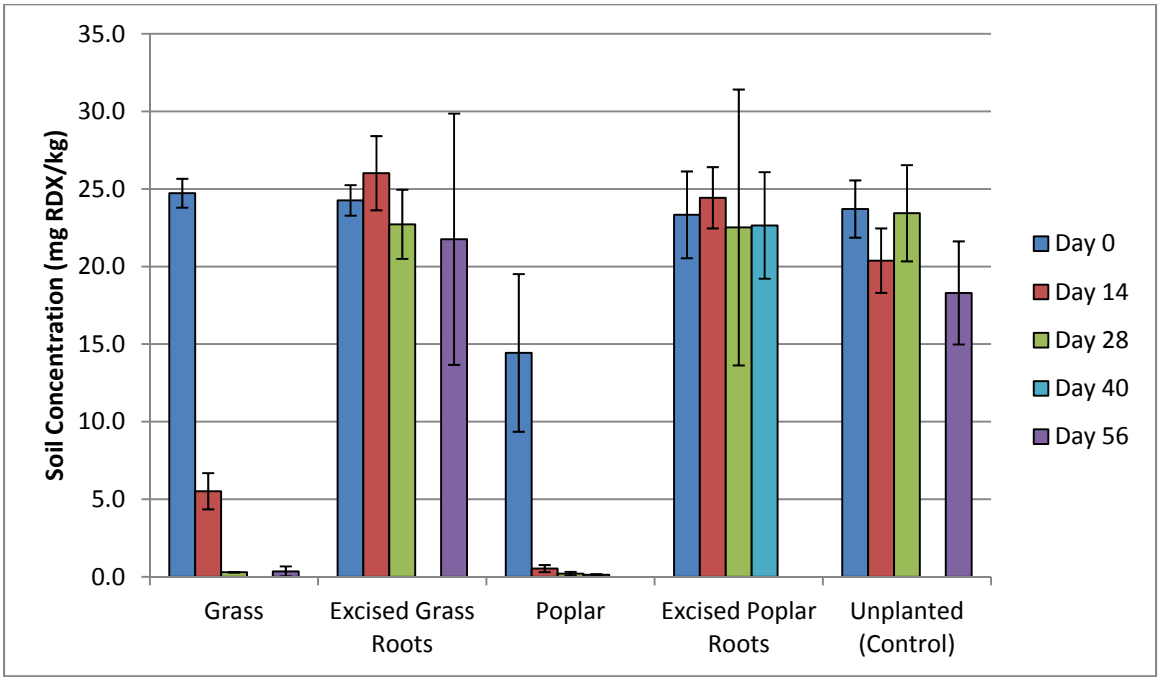


Figure 39. RDX concentration in soil of each microcosm.

The reduction of RDX concentrations in soil was expected given past research that has shown the translocation of RDX to plant tissues in many plant species (Just & Schnoor, 2004;

Harvey, Fellows, Cataldo, & Bean, 1991; Thompson & Schnoor, 1997; Larson, Jones, Escalon, & Parker, 1999). The objective of the experiment was to verify the reduction in concentration of RDX in native Eglin AFB soil. This objective has been completed with positive results. The result of zero detections in the blade or leaf of the plants contradicts literature which shows RDX translocation primarily to the leaves or blade of the plant (Brentner et al., 2009). Multiple transformation processes observed following the uptake and translocation to the leaves has also been demonstrated (Van Aken, Yoon, Just, & Schnoor, 2004). The analysis method used was only designed for RDX, no RDX metabolites would have been detected.

The lack of RDX degradation in the excised root microcosms suggest the plant exudates and/or root decomposition have little to no effect over a time period of 56 days. The result is supported by work previously completed (Anderson, 2010) where it was found that the naturally occurring microbial communities in Eglin AFB Lakeland soil did not have the capacity to degrade RDX.

Bioavailability of TNT and RDX in Soil from Eglin AFB

Sorption and desorption studies of TNT to Dorovan muck, Lakeland soil, and hybrid poplar root and leaf tissue were conducted according to the equilibrium batch method. Sorption isotherms were nonlinear following a Freundlich-type relationship for TNT with Dorovan muck, Lakeland soil, and poplar root tissue. The sorption isotherm was linear for TNT with poplar leaf tissue. Sorption capacities (K_d) follow the order of magnitude of muck soil >> poplar root tissue > leaf tissue >> sandy soil (see Table 7). Overall, the results indicate that the sorption capacity in muck soil is substantially greater than that in sandy soil. Therefore, sorption in muck soils may limit initial mobility.

Table 7. Sorption capacities (K_d) from equilibrium batch method.

Sorbent Processes	Regression Equation	R^2	K_d (L/kg)		
(Q: mg/kg, Ce: mg/L)			Ce = 0.01	Ce = 0.1	Ce = 10
Dorovan Muck Sorption	logQ = 0.850 logCe + 2.755	0.984	1133	803	403
First Desorption	logQ = 0.868 logCe + 2.965	0.991	1693	1250	682
Second Desorption	logQ = 0.866 logCe + 3.153	0.990	2638	1937	1045
Lakeland Soil Sorption	logQ = 0.816 logCe + 0.416	0.989	6.08	3.98	1.71
First Desorption	logQ = 0.829 logCe + 0.819	0.987	14.48	9.77	4.44
Second Desorption	logQ = 0.856 logCe + 1.066	0.990	22.63	16.23	8.35
Poplar Root Sorption	logQ = 0.882 logCe + 1.848	0.997	121.4	92.49	53.64
First Desorption	logQ = 0.868 logCe + 1.933	0.994	157.2	116.1	63.37
Second Desorption	logQ = 0.869 logCe + 2.052	0.991	206.2	152.4	83.29
Poplar Leaf Sorption	Q= 50.34 Ce + 6.307	0.999	50.34	50.35	50.34

Substantial desorption hysteresis was apparent for TNT on Dorovan muck and Lakeland soil, as manifested by much higher K_d values for desorption than for sorption (see Figure 40 and Figure 41). Weak desorption hysteresis was observed for TNT on poplar tree root tissue (see Figure 42). The intensity of desorption hysteresis increased with increased iterations of the desorption process. The large difference in the sorption and desorption K_d values demonstrates that a significant fraction of the TNT was irreversibly sorbed or subject to very slow desorption kinetics during the test. However, there is little desorption hysteresis of TNT on poplar tree leaf tissue. This indicates that it is a reversible sorption-desorption process (see Figure 43). Significant sorption-desorption hysteresis of TNT in soils would limit bioavailability of TNT to microbes and plants. Weak or little sorption-desorption hysteresis of TNT in plant root and leaf tissue would allow for potential ecotoxic effects in nature due to leaching of TNT from leaf litter and root turnover after uptake and accumulation from soil.

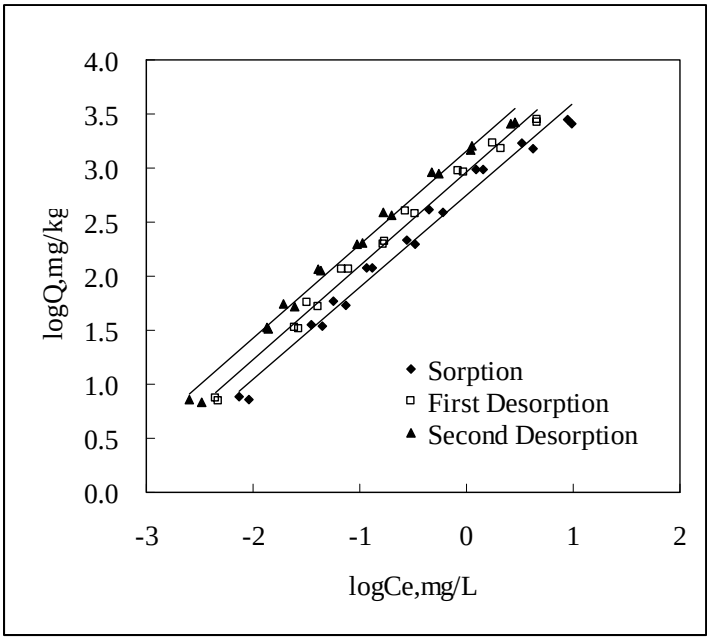


Figure 40. Sorption-desorption isotherms of TNT with Dorovan muck soil.

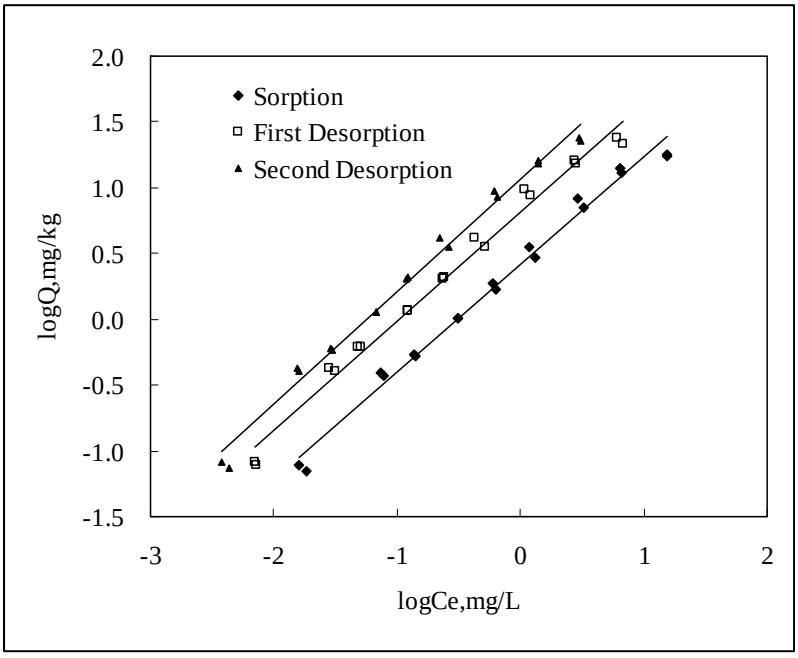


Figure 41. Sorption-desorption isotherms of TNT with Lakeland (sandy) soil.

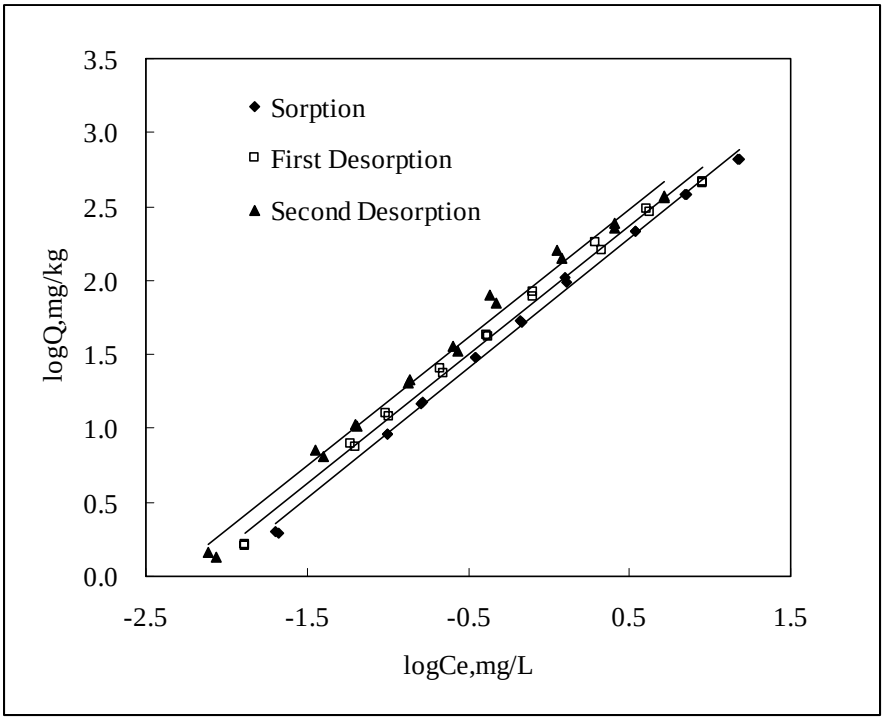


Figure 42. Sorption-desorption isotherms of TNT with poplar tree root tissue.

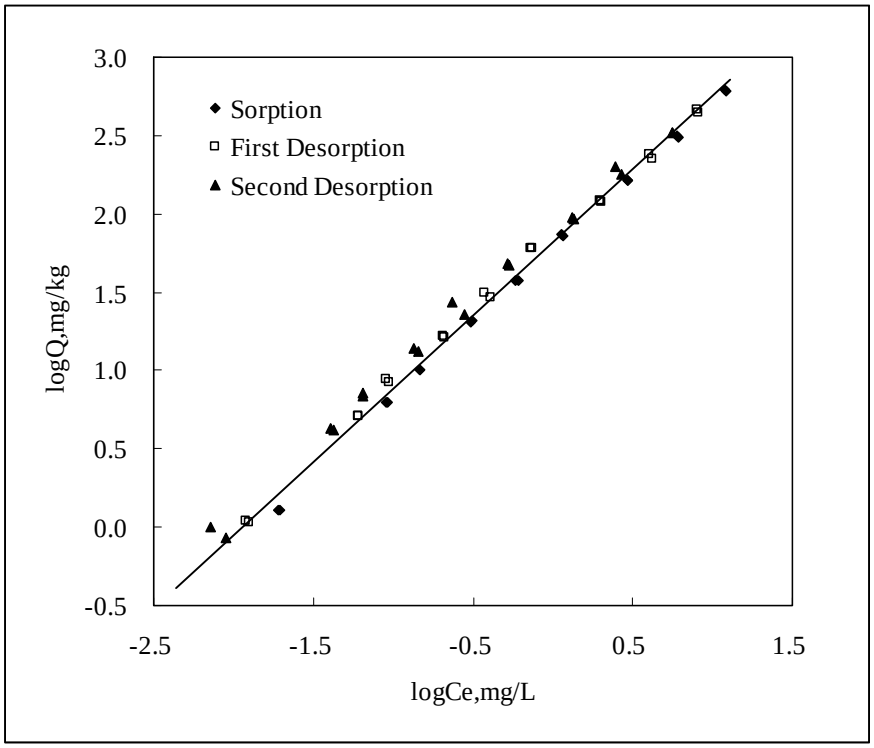


Figure 43. Sorption-desorption isotherms of TNT with poplar tree leaf tissue.

Sorption of RDX to Dorovan muck soil followed a Freundlich isotherm, with K_d = 57.7 L kg^{-1} (see Figure 44). Sorption of RDX to Lakeland soil did not follow a Freundlich isotherm, but fit a Langmuir model. Sorption capacity of RDX to Lakeland soils was not significant at field aqueous concentrations. RDX is expected to be fairly mobile and therefore bioavailable in these soil systems.

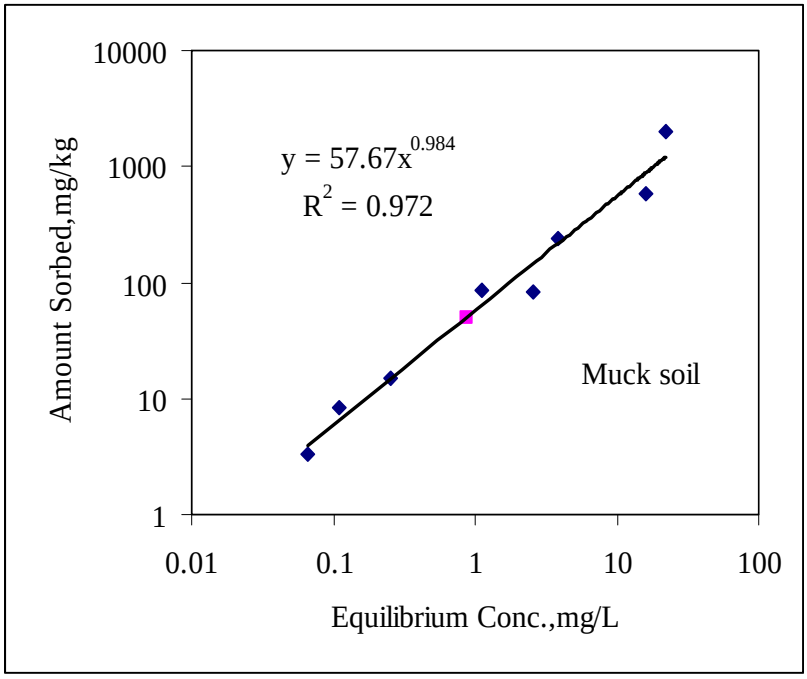


Figure 44. Sorption-isotherm of RDX with Dorovan muck soil.

Analysis of TNT in soil samples from the potted plant study provides evidence of the effect of aging on the bioavailability of TNT in these systems. Dorovan muck soils aged with TNT at 4°C for 6 months have much slower and more incomplete TNT removal than soils that were freshly contaminated with TNT at the onset of the experiment. No significant difference was identified between aged and freshly contaminated Lakeland (sandy) soils. It is expected that bioavailability would be more markedly affected by aging in muck soils because the relatively high fraction of organic carbon and higher cation exchange capacity, both characteristic of soils to which TNT will adsorb irreversibly to as demonstrated in earlier sorption studies. The sorption capacity of TNT in sandy soils is much lower and reversible. Analyses of radioactivity in filter residues from soil extracts indicate that the "unextractable" fraction of TNT is similar between aged and freshly contaminated soils and between soils types. On average, about 2% of the initial activity from [U-^{14}C]-TNT spiked in Dorovan muck soils and 3% in Lakeland soils was not extractable by acetonitrile using a standard TNT extraction method.

In order to analyze how aging of RDX in soils affects soil composition, soil samples were studied using Mid-IR spectroscopy with an ATR accessory. The instrument was set up for 32 co-added scans with a resolution of 4 cm^{-1} and a scanning range of 4000-500 cm^{-1}. Four RDX-spiked samples were studied: freshly contaminated or aged (one year) sandy or muck soil. The samples were dried and stored at room temperature. Large chunks of the soil samples were gently pressed to separate and to ensure a homogeneous spread of sample parts on the ATR diamond crystal. The results indicate that the compositions of both fresh and aging mucky soils

are identical as concluded from their superimposed spectra. Fresh and aged sandy soils showed a slight difference in chemical composition as well as absorbance values at approximately 1800 cm^{-1}. Changes in chemical composition may reflect the presence of stable soil-RDX complexes formed during the aging process.

Task 4. Metabolite and Pathway Identification

Gene Expression by Hybrid Poplar Exposed to TNT

Hybrid poplar tree cuttings (*Populus deltoides x nigra*, DN34) were exposed to 2,4,6-trinitrotoluene (TNT) with a concentration of 5 mg L^{-1} TNT. RNA extraction from exposed cuttings and negative controls were done at 8, 24, and 48 hours after exposure. RNA samples were hybridized to Affymetrix GeneChips® for genetic analysis. Statistically significant expressed genes were implicated in all three main phases of uptake, transformation, and sequestration of TNT.

Plant uptake and metabolic transformation of xenobiotic compounds involves three main phases (Madhou *et al.*, 2006). Phase one involves the transformation of the parent compound into a more soluble and typically less toxic daughter product. In phase two, conjugation begins with an increase in metabolic activity that is catalyzed by glycosyl-, malonyl-, and glutathione s-transferases. In the final phase, the conjugated compound is transported into the vacuole or cell wall from the cytosol via ATP-binding, ABC transporters and multi-drug resistant proteins for sequestration or compartmentalization. This study incorporates these phases as an outline to understand the genetic responses of the hybrid poplar tree to trinitrotoluene (TNT).

In phase one, TNT is degraded in a series of steps involving nitroreductases, monooxygenases, and, more specifically, cytochrome P450's. Previous Arabidopsis research has focused primarily on the cytochrome P450's, but has included several unknown nitroreductases and other monooxygenases also implicated in this phase of transformation (Madhou et al., 2006; Rylott et al., 2006). Over the 48 hours of this study, cytochrome P450's were the primary gene superfamily upregulated with respect to phase one. As indicated in Table 8 and Figure 45, the expression trend of these genes was noticeable beginning at 8 hours, and it peaked around 24 hours typically declining in upregulation by 48 hours. The range of expression was between 2-fold and 4-fold greater than the control cuttings for those same times. This is consistent with previous findings that poplar tree cuttings take up TNT quickly, usually within 10 hours, and immediately begin transformation (Thompson et al., 1998).

Table 8. List of phase 1 Cytochrome P450 genes (by Affymetrix ID) upregulated at 8, 24, and 48 hours after exposure to TNT.

Affymetrix Probe ID	8 h	24 h	48 h	Molecular & Biological Functions	Gene Type
PtpAffx.132618.1.S1_at		3.16	2.55	Monooxygenase Activity; Electron Transport	Cytochrome P450
PtpAffx.138857.1.A1_at	3.31	3.75	3.13		
PtpAffx.151850.1.A1_at		2.45	2.13		
PtpAffx.222544.1.S1_at		2.43	3.28		
PtpAffx.225096.1.S1_s_at			2.05		
PtpAffx.33300.1.A1_at	2.25	3.36	3.27		
PtpAffx.35496.1.A1_at		2.57	2.37		
PtpAffx.90126.1.S1_at	2.19	3.11	3.11		

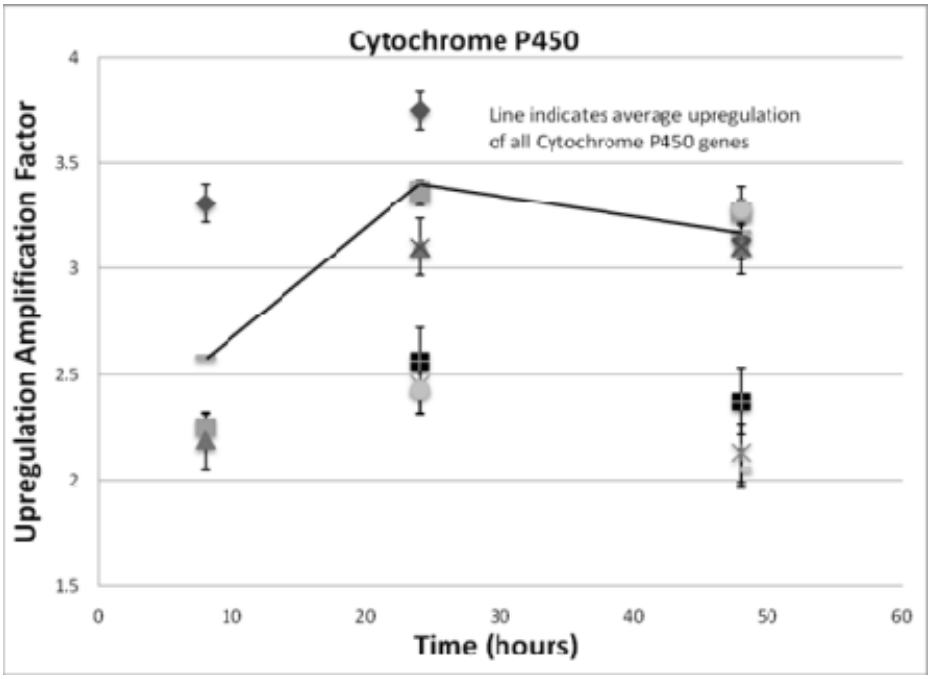


Figure 45. Upregulation of Cytochrome P450s at 8, 24, and 48 hours. The average of all CytP450 genes is plotted as a line and standard deviation error bars are shown. Cytochrome P450 genes are identified by Affymetrix® ID in Table 8.

During the transformation of the nitro groups, cupin was also significantly upregulated, ranging between 2-fold and 5-fold upregulation in gene expression (Table 9). Cupin is a nutrient reservoir for nitrogen, which would increase in concentration as transformation continued, suggesting that the plants are utilizing nitrogen in the nitro-moieties as a nitrogen-nutrient source. Not much has been discussed in Arabidopsis studies with respect to cupin, but we found it to be one of our most significantly upregulated genes. Cupin and nitrogen reservoir upregulation accounted for around 11% of the identifiable significantly upregulated genes. This response could be important in determining new pathways for the transformation and nutrient uptake of nitro-substituted xenobiotics.

Table 9. Upregualtion of Cupin during phase 1.

Affymetrix Probe ID	8 h	24 h	48 h	Molecular & Biological Functions	Gene Type
PtpAffx.211043.1.S1_at	2.54	2.93	2.62	Nutrient reservoir activity	Cupin
PtpAffx.211091.1.S1_s_at	4.05	5.13	5.63		
PtpAffx.217785.1.S1_at			2.01		
PtpAffx.3487.1.S1_at			2.59		

In phase two, metabolic activity began to increase as TNT was deactivated by conjugation. This deactivation can be catalyzed by glycosyl-, malonyl-, and glutathione s-transferases. The most common of these transferases, glutathione S-transferases (GSTs), has been reported in most TNT related studies. Some recent studies have also reported glycosyl- and

glucosyl-transferase activity as a part of phase two conjugation (Weis et al., 2006). This study also found upregualtion of GST, glycosyl- and glucosyl-transfereases, but no significant malonyl-transferase upregulation. These results are reported in Table 10 and Figure 46 through Figure 48.

Table 10. List of phase 2 gene upregulation of GST's, glycosyl- and glucosyltransferases.

Affymetrix Probe ID	8 h	24 h	48 h	Molecular & Biological Functions	Gene Type
Ptp.5289.1.S1_s_at	2.03	2.43	2.28	Metabolism	Glutathione S-transferase (GST)
PtpAffx.224201.1.S1_at	2.07	3.80	2.06		
PtpAffx.224631.1.S1_s_at	2.38	2.26	2.78		
PtpAffx.4246.2.S1_a_at	3.38	3.44	3.34		
PtpAffx.43231.1.A1_at	3.26	3.29	3.63		
Ptp.3709.1.S1_at	2.99	3.27	2.55		
PtpAffx.203138.1.S1_at			2.49	Hydrolase activity, Hydrolyzing O-glycosyl compounds	Glycosyl transferase
PtpAffx.223848.1.S1_at			2.03		
Ptp.3522.1.S1_s_at			2.00		
Ptp.6729.1.S1_at		2.44	2.40		
PtpAffx.127059.1.A1_at	2.47	2.97	3.66		
PtpAffx.200185.1.S1_s_at	2.27	3.31	2.58		
PtpAffx.209038.1.S1_s_at	2.26	2.54	2.52		
PtpAffx.217237.1.S1_at	4.46	3.86	2.17		
PtpAffx.221654.1.S1_s_at		2.42	2.05		
PtpAffx.224410.1.S1_at	2.34	2.80	2.13		
Ptp.4506.1.S1_s_at	4.09	4.66	2.64		
PtpAffx.32356.3.S1_at	3.83	4.95	2.64		
PtpAffx.83041.1.A1_s_at	3.07	3.62	2.56		
Ptp.6958.1.S1_s_at	2.29	2.76	2.05	Metabolism	UDP-glucuronosyl/ UDP-glucosyltransferase
PtpAffx.139063.1.S1_at	2.40	3.32	3.27		
PtpAffx.215169.1.S1_s_at	3.64	4.45	4.32		
PtpAffx.216568.1.S1_s_at	2.49	3.93	4.82		
PtpAffx.224189.1.S1_at	4.00	4.26	4.00		
PtpAffx.22666.1.A1_s_at		3.30	4.41		
PtpAffx.2665.1.S1_at		2.50	2.19		
PtpAffx.31211.1.A1_at		2.04	2.22		
PtpAffx.23657.1.S1_s_at	2.05	2.47	3.59		
PtpAffx.23657.2.S1_s_at	2.02	2.56	3.74		
PtpAffx.158231.1.A1_at	2.26	2.78	2.55		

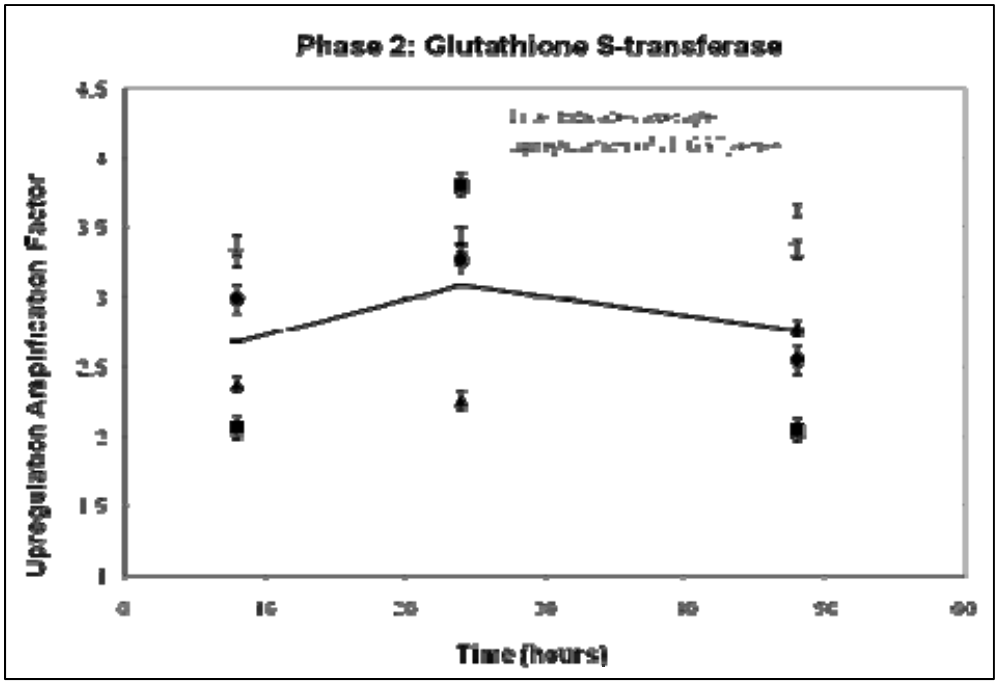


Figure 46. Phase 2 plots of GSTs over time, with standard deviation error bars. Line represents the average upregulation of the gene.

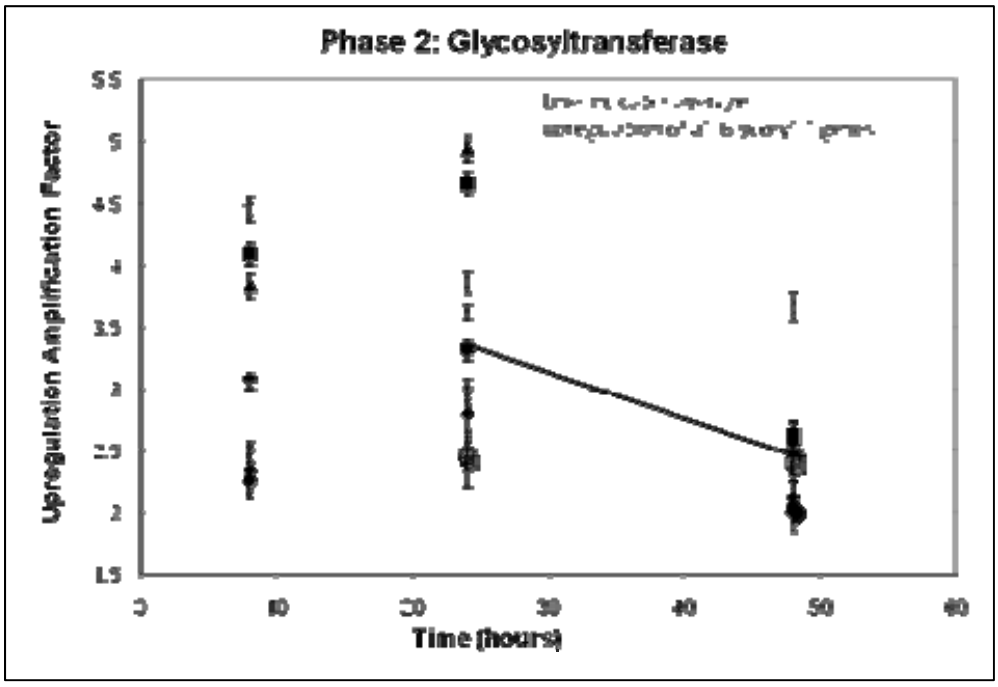


Figure 47. Phase 2 plots of Glycosyltransferases over time, with standard deviation error bars. Line represents the average upregulation of the gene.

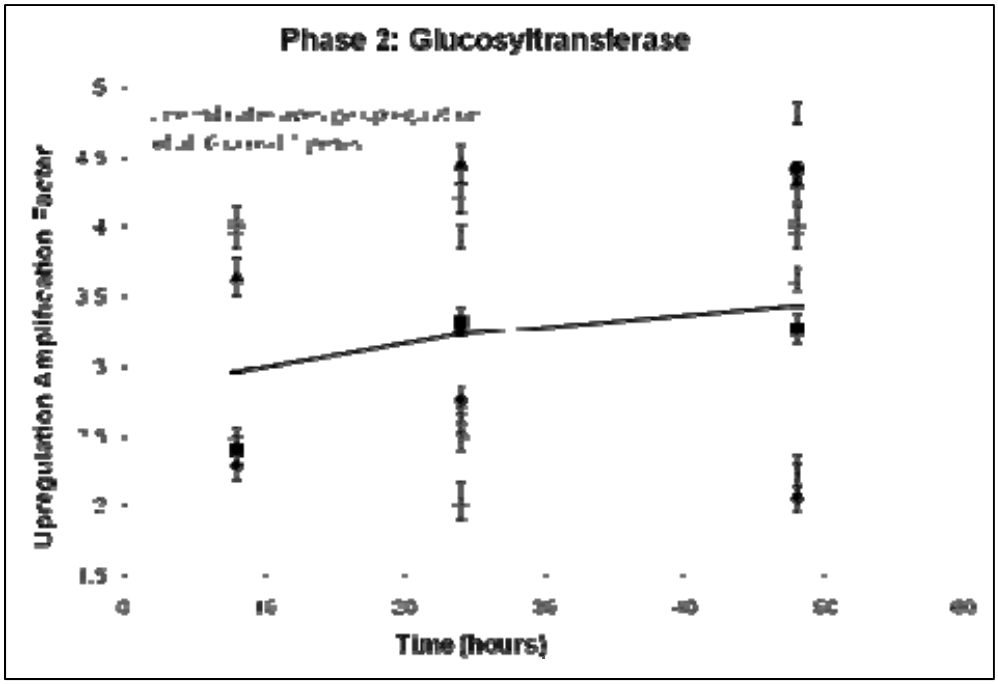


Figure 48. Phase 2 plots of Glucosyltransferases over time, with standard deviation error bars. Line represents the average upregulation of the gene.

GST and glycosyltransferase both followed similar patterns to the cytochrome P450's over the course of this experiment. Results indicated upregulation in the range of 2-5 fold from the poplar controls, and they reached their peak expression at approximately 24 hours. Glucosyltransferases, however, did not follow the same trend (see Figure 48). Their average expression indicated a steady increase with a possible tapering-off just past 24 hours. Some of the genes began to return to a lower level of expression during the time course, while others maintained a high expression. This would be consistent with phase two conjugation following a slight time lag after phase one transformation by P450s.

Phase three involved the transport of the conjugated TNT products from the cytosol into the vacuole or cell wall by transport proteins. This transport is typically marked by an increase in ATP-binding/ABC transporter activity and multi-drug resistance (MDR) proteins. For our study, we found all of these genes were significantly expressed in the 24-48 hour time periods (see Table 11). No significant expression was observed at the 8-hour time period, and very little upregulation was noted at the 24 hour sampling time. Most of the significant upregulation occurred between 24 and 48 hours and corresponded to the gene superfamily of protein transporters. In addition to the ATP, ABC and MDRs, many other transporter proteins were also upregulated at the 48 hour sampling time.

Table 11. List of phase 3 upregulated genes.

Affymetrix Probe ID	8 h	24 h	48 h	Molecular & Biological Functions	Gene Type
PtpAffx.208904.1.S1_at		2.24	4.07	Antiporter activity; Membrane	Multidrug transporters
PtpAffx.208905.1.S1_at			3.88		
PtpAffx.213048.1.S1_at			2.58		
PtpAffx.212897.1.S1_s_at			2.28	ATP-binding cassette; ABC transporter activity	Membrane transport; ABC transporter
PtpAffx.214961.1.S1_s_at		2.40	2.39		
PtpAffx.225122.1.S1_s_at			3.37		
PtpAffx.225531.1.S1_s_at			2.01		
PtpAffx.225717.1.S1_s_at			2.27		
Ptp.2887.1.S1_at			4.10		
PtpAffx.114367.1.A1_at			2.56		
PtpAffx.141628.1.S1_at			3.81		
PtpAffx.202418.1.S1_at		2.22	2.69		
PtpAffx.218920.1.S1_s_at			3.39		
PtpAffx.22198.1.S1_at			3.35		
PtpAffx.223705.1.S1_x_at			4.21		
PtpAffx.61049.1.S1_at			3.09		
PtpAffx.87677.1.S1_at			2.94		
Ptp.7558.1.A1_at		2.01	2.32	Transporter activity; Membrane	Adenine nucleotide translocator 1
PtpAffx.14146.1.A1_s_at		2.01	2.45		
PtpAffx.159072.1.S1_at			2.76		
PtpAffx.83667.1.A1_at			2.95		
PtpAffx.204923.1.S1_at			2.20	Oligopeptide transport; Membrane	TGF-beta receptor, type I/II extracellular region
PtpAffx.204923.1.S1_x_at			2.03		
PtpAffx.216706.1.S1_at	2.22	3.21	3.98		
PtpAffx.216706.1.S1_x_at	2.20	3.16	3.92		
PtpAffx.75314.1.A1_at			2.31		
PtpAffx.221953.1.S1_at			2.17	Transporter activity; Membrane	Plasma membrane intrinsic protein
PtpAffx.221953.1.S1_s_at			2.43		
Ptp.4477.1.S1_at			2.21		
Ptp.1224.1.S1_s_at			2.27		Lipocalin-related protein

Additional genes groups that were induced at greater than 2-fold expression values are shown in Figure 49. Metabolic processes accounted for 30% of the significantly upregulated genes in this experiment. Previously discussed genes involved in the three main phases are located in the “other” expression group that makes up about 24% of the significantly expressed genes. Standard categories of biosynthesis, transport, and binding account for the rest of the genes significantly expressed compared to the control plants.

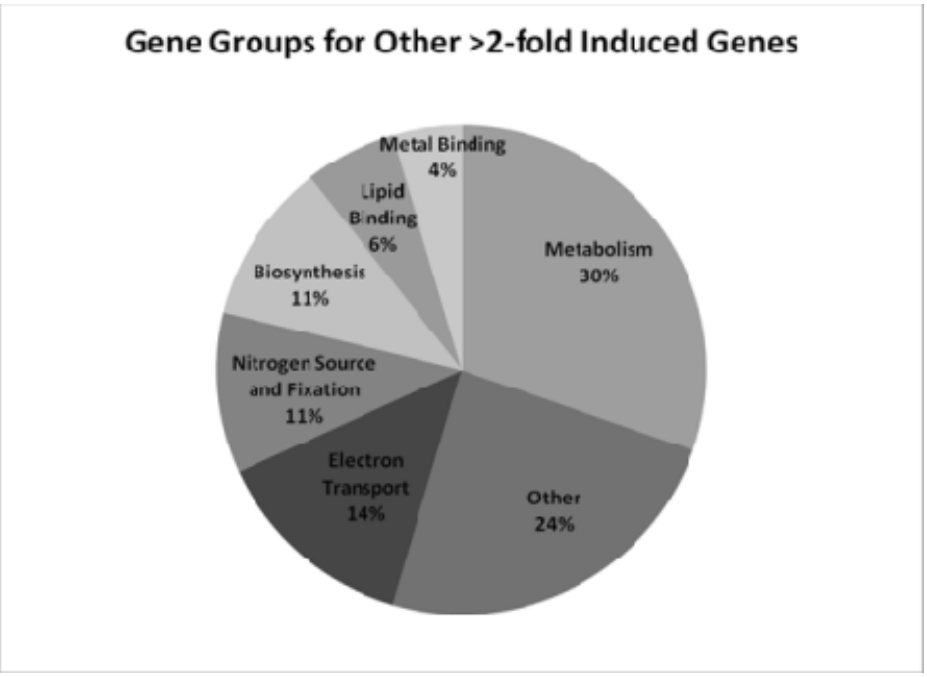


Figure 49. Chart of gene categories for other significantly upregulated genes over the course of 48 hours when exposed to TNT.

In order to develop a more complete, global understanding of the poplar tree's response to TNT, we also looked at those genes that were significantly downregulated during the 48 hours of this study. Some of these genes are of interest, as they play contrary roles in the processes previously discussed for phases 1-3. Other downregulated genes from this study have previously been reported to be significant in the areas relating to energy regulation, respiration, transcription, translation, and growth. Of the 286 significantly downregulated genes, around 14% were downregulated at the 8-hour sampling time. This doubled to around 30% at the 24-hour sampling time. At the 48-hour sampling time we saw the largest combination of gene repression comprising 82% of the significantly downregulated genes. Whereas gene expression peaked primarily in the 24 to 48 hour sampling range, as predicted, the downregulation of genes did not exhibit an overall peak time. As downregulation is often a response to other factors, it may be that extended sampling times would help clarify the importance of some of the more significantly downregulated genes reported here.

One pattern of significant downregulation can be correlated to the process of respiration. As the metabolism of the plant increased to compensate for the phase two conjugation of the TNT metabolites, downregulation of respiration-related genes became obvious. Of the 286 downregulated genes, 73 were respiration process related. Initially, we noticed that several phosphofructokinases (PFKs) had been downregulated from the controls ranging between -2.9 and -5.7 fold changes. The 8 and 24-hours sampling times revealed about a -3.2 fold decrease in PFK expression. At 48 hours this had changed to an average -5.5 fold change in response from the controls. This reduction of PFKs during an increase in metabolism has been shown to occur in previous plant studies, but has not been reported in poplar tree experiments. PFKs are considered to be an important part of the rate-limiting step of glycolytic flux during periods of increased metabolic activity (Winkler et al., 2007). PFKs and other pyrophosphates relate directly to glycolysis, which an integral part of cellular respiration.

This pattern of significant downregulation in respiration related genes continued with key genes involved in the steps or pathways leading to the citric acid cycle. These include pyruvate kinases, hexokinases, phyrophosphorylases, fatty acid desaturases, acetyl co-A enzymes and aspartate kinase. The most commonly downregulated repiration related activity in this study was the protein-tyrosine kinase activity and protein serine/threonine kinase activity. Protein-tyrosine is an intermediate on the shikimate pathway of phosphorylation and plays a key role in plant metabolism. Peroxidases were also downregulated as seen in Table 12, which has previously been reported to be important with respect to lignin content and composition in hybrid aspen trees as well as a response to oxidative stress (Li et al., 2003).

Table 12. List of downregulated genes in response to oxidative stress.

Affymetrix Probe ID	8 h	24 h	48 h	Molecular & Biological Functions	Gene Type
Ptp.719.1.S1_at			-2.18	Peroxidase activity	Response to oxidative stress
PtpAffx.10355.2.S1_at	-4.43	-4.10	-5.21		
PtpAffx.16117.1.A1_a_at		-2.31			
PtpAffx.18226.1.A1_a_at			-2.34		
PtpAffx.211356.1.S1_at	-2.56	-3.29	-3.30		
PtpAffx.213709.1.S1_at		-2.86	-2.84		
PtpAffx.216844.1.S1_at			-2.42		
PtpAffx.224549.1.S1_s_at			-2.03		
PtpAffx.225157.1.S1_at			-2.53		
PtpAffx.5.2.S1_a_at	-2.57	-3.32			

Significant downregulation implies the cell was over stimulated by this gene family for a period of time and the expression of that receptor protein needed to be decreased to protect the cell. This downregulation pattern is likely indicative of an increase in respiration due to the plant's phytotoxic response to stress. This is supported by the overall percentage of downregulation relating to both respiration (see Figure 50) and the more specific pathways of shikimatic acid and glycolysis in response to the conjugation of the xenobiotic in the cell.

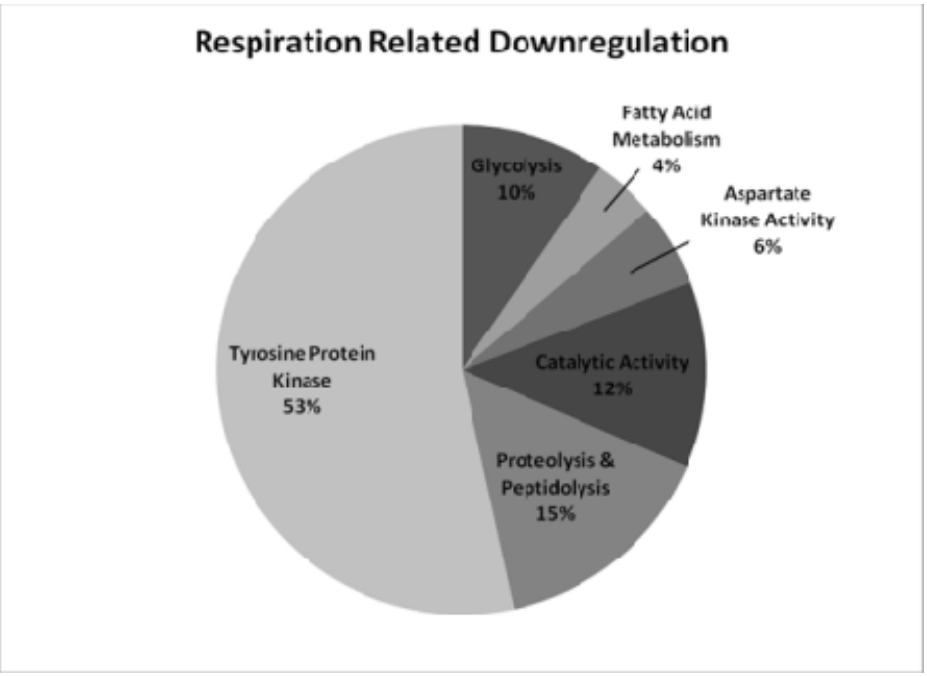


Figure 50. Chart of gene functions of 73 respiration related downregulated genes.

During phase three, transport proteins carry large conjugated organic molecules into the vacuole for sequestration. As this transportation occurs, pumps extrude calcium ions into the cytosol as a balancing mechanism. When there is an abundance of these ions in the cell, downregulation begins in order to maintain homeostasis. This research found eleven genes encoding for calcium ion binding that were significantly downregulated at the 48 hour sampling time. This information supports the hypothesis that TNT is being sequestered in the cell wall during phase three.

Another pattern of downregulation in the area of transcription, RNA processing, translation and posttranslation was also identified. Genes encoding for methylation, targeting endoplasmic reticulum (ER) and DNA binding, including helix-turn-helix, zinc fingers, helix-loop-helix, leucine zippers and basic zippers, were significantly downregulated (Figure 5). These are commonly downregulated genes and support the consistency of this research to previous findings in this area.

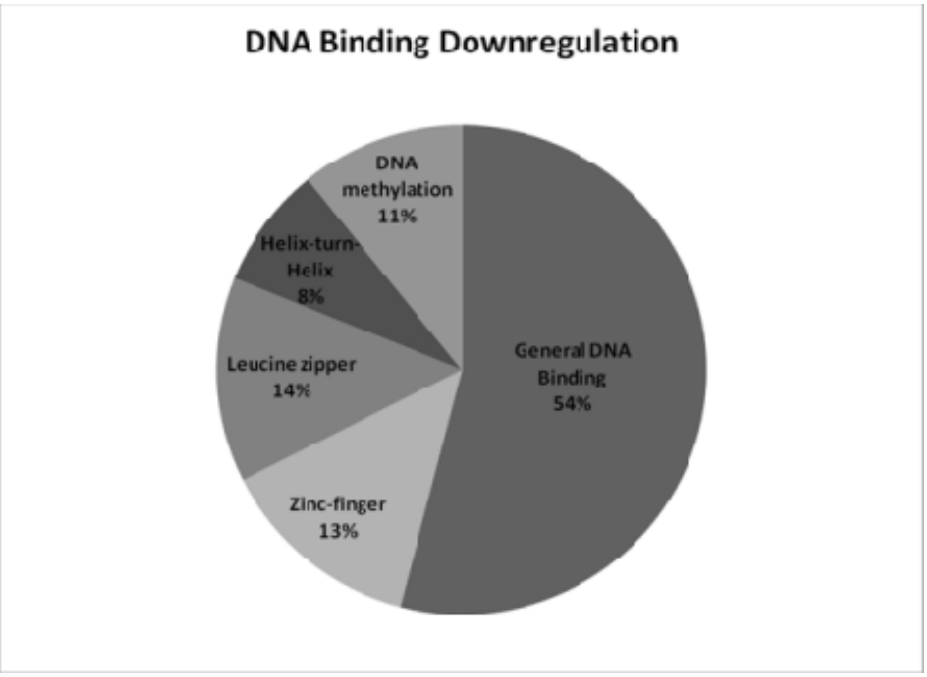


Figure 51. Chart of gene functions of 37 DNA Binding related downregulated genes.

In addition to these observable patterns, other genes were significantly repressed. This study found a large number of heat shock proteins were downregulated in our samples. This is common for toxic responses in plants and serves only to confirm that our results are consistent with previously observed phytotoxic response mechanisms. Heat shock proteins reflect the general toxicity of TNT to Populus, but they are likely not involved in the actual transformation and detoxification of TNT.

Characterization of Microbial Community in Soils from Eglin AFB

The microbial community present in Lakeland Soil and Dorovan muck samples was determined using Terminal Restriction Fragment Length Polymorphism (T-RFLP). T-RFLP has been shown to be a powerful tool for assessing the diversity of complex bacterial communities and for rapidly comparing the community diversity of different ecosystems (Liu, Marsh, Cheng, & Forney, 1997). T-RFLP is also a more appropriate microbial fingerprinting technique than Denaturing Gradient Gel Electrophoresis (DGGE) for large sample numbers due to its greater reproducibility (Smalla et al., 2007). The terminal restriction fragments of each 16S rRNA gene created during the T-RFLP method provides a quantitative basis for estimating diversity that is more sensitive than other techniques in microbial ecology (Tiedje, Asuming-Brempong, Nusslein, Marsh, & Flynn, 1999).

T-RFLP is a culture-independent method of obtaining the genetic fingerprint of a microbial community. During the T-RFLP method, extracted DNA is amplified through Polymerase Chain Reaction (PCR) using a fluorescent primer. The fluorescent molecule attached to the primer is tagged to one end of the PCR amplicons during the PCR process. The amplified PCR product is then digested using restriction enzymes, producing terminal restriction fragments (fragments which are tagged with a fluorescent molecule at their terminal end). The terminal restriction fragments (T-RFs) are then separated by electrophoresis providing their size in base pairs and intensity of fluorescence. T-RF sizes can then be compared to known

sequences in databases for phylogenetic assignment and analysis of microbial community (Blackwood, Marsh, Kim, & Paul, 2003).

T-RFLP has been used successfully for the comparison of bacterial diversity and composition in environmental soil samples (Hackl, Zechmeister-Boltenstern, Bodrossy, & Sessitsch, 2004). T-RFLP has also been implemented for monitoring the spatial and temporal variations in the microbial structure of agricultural soil (Lukow, Dunfield, & Liesack, 2000). This relatively new technique may be an important addition to more completely characterizing remediation efforts at contaminated sites.

The relative abundance of the microbial community present in Lakeland Soil at the family and phylum taxonomic level is provided in Figure 52 and Figure 53, respectively. There were 5 phyla, 8 classes, 13 orders, and 19 families classified in Lakeland Soil. As shown in Figure 52, the dominant family in Lakeland Soil is Burkholderiaceae, a Betaproteobacteria which represents 68% of the relative abundance at the family level. The dominant phylum present was proteobacteria which represented 97% of the relative abundance at the phylum level. Nearly 90% of the relative abundance was represented by only three families of bacteria, indicating that the microbial community lacks significant diversity in Lakeland Soil.

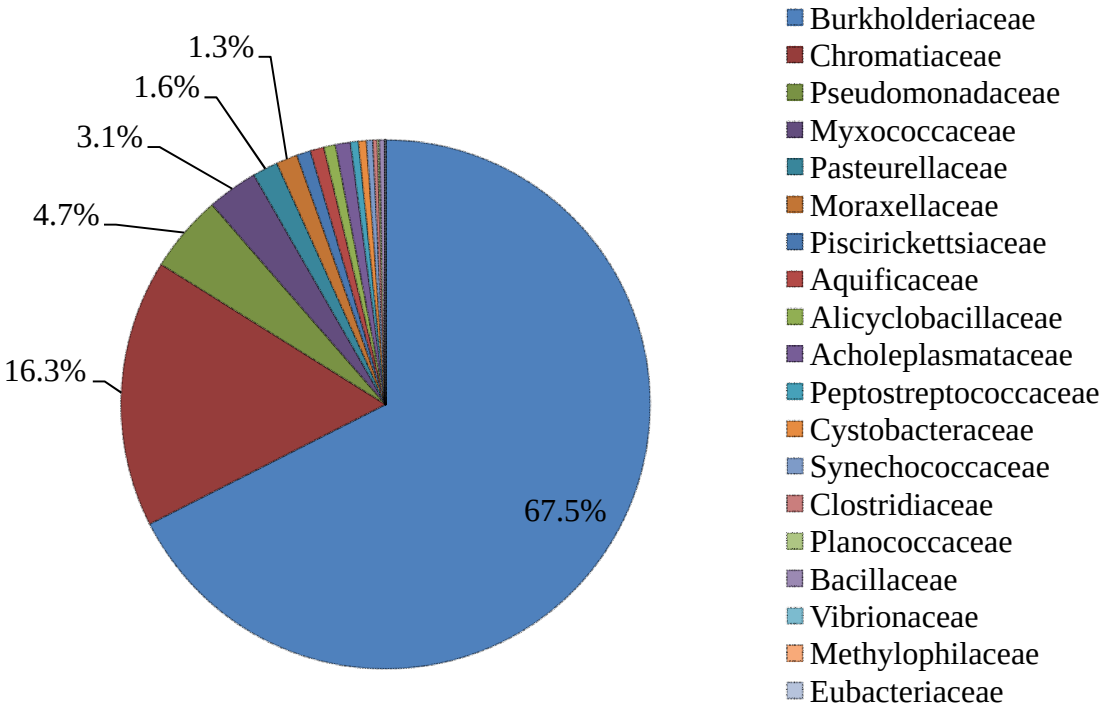


Figure 52. Microbial community of Lakeland Soil at the family classification level.

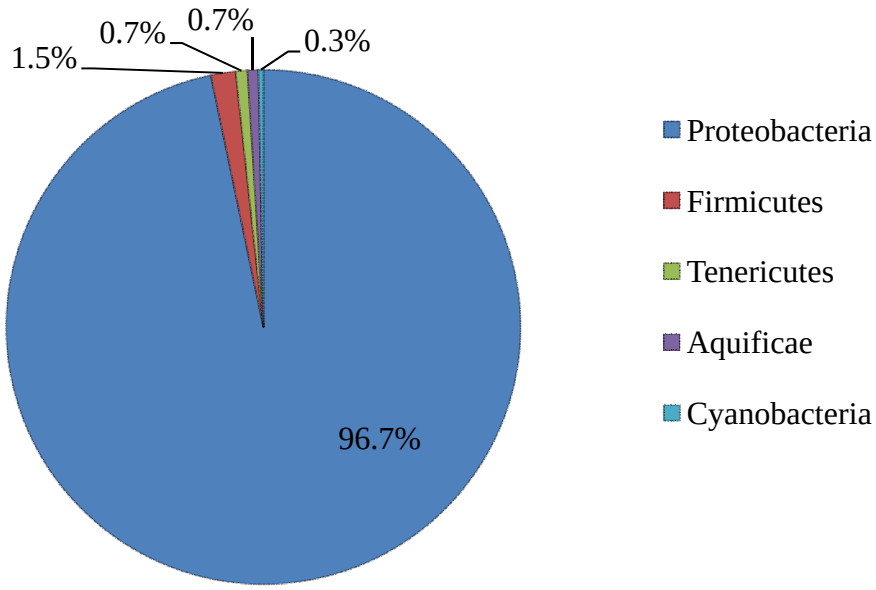


Figure 53. Microbial community of Lakeland Soil at the phylum classification level.

The relative abundance of the microbial community present in Dorovan muck at the family and phylum taxonomic level is provided in Figure 54 and Figure 55, respectively. Dorovan muck contained nearly twice as many phylogenetic classifications as Lakeland Soil at every level, with 10 phyla, 12 classes, 26 orders, and 43 families. The relative abundance was also more evenly distributed among different taxonomic levels than in Lakeland Soil. As shown in Figure 54, the dominant family in Lakeland Soil is Rhodobacteraceae, an Alphaproteobacteria. Dorovan muck was similar to Lakeland Soil in that proteobacteria heavily dominated the microbial diversity by representing 64% of the relative abundance at the phylum level. Cyanobacteria, also known as blue-green algae, were also found in Dorovan muck and represented 6.6% of the relative abundance at the phylum level. The significant presence of cyanobacteria is likely due to the location from which the Dorovan muck was sampled, which was a dried swamp bed.

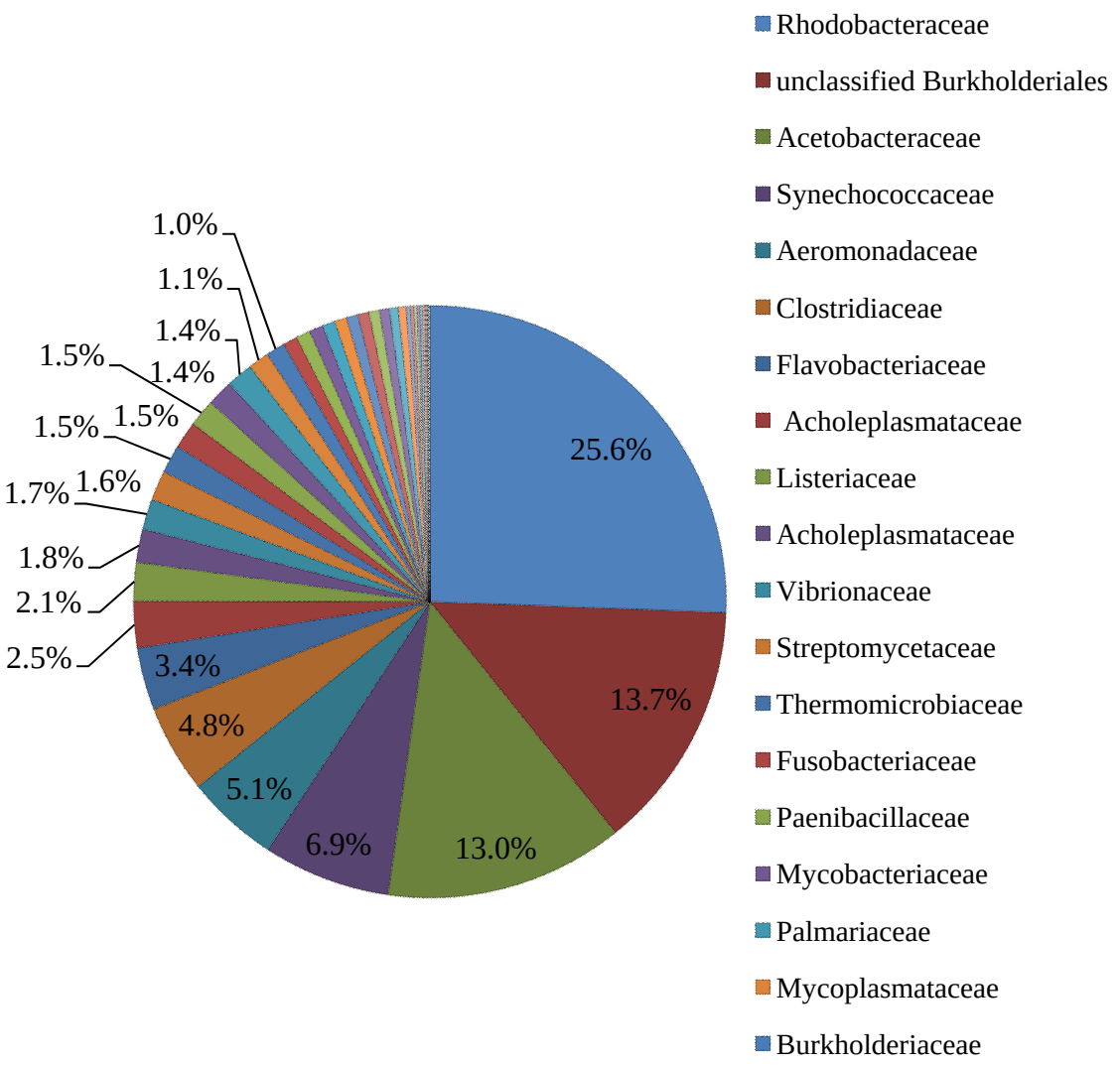


Figure 54. Microbial community of Dorovan muck at the family classification level. Only families with 1% or higher relative abundance are shown in the legend.

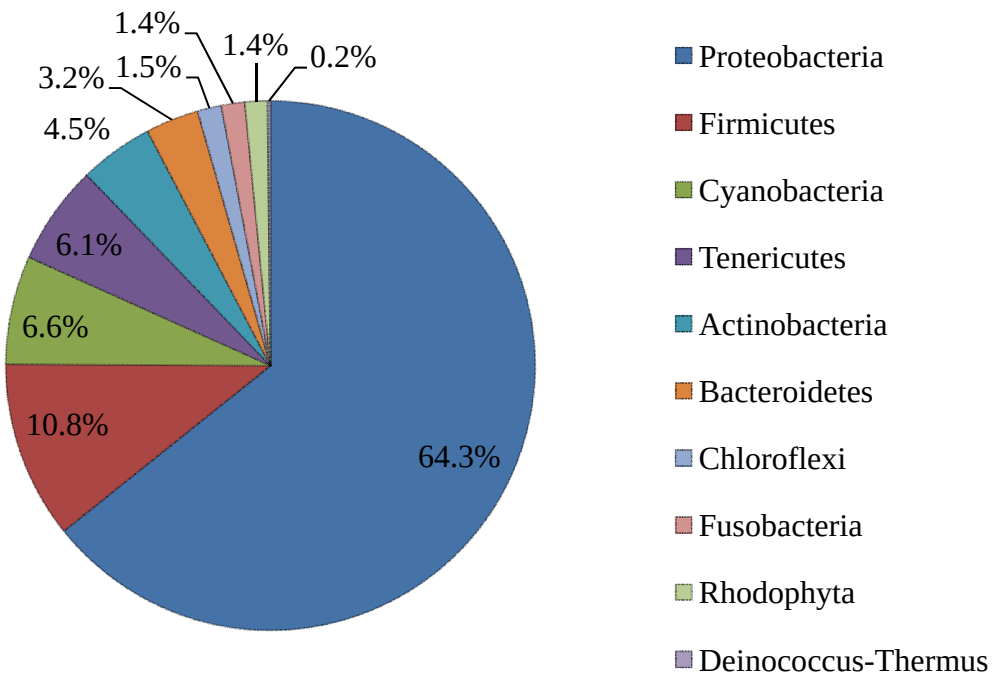


Figure 55. Microbial community of Dorovan muck at the phylum classification level.

The microbial community of Dorovan muck was much more diverse than that of Lakeland Soil. The higher levels of organic carbon and nutrients present in Dorovan muck are likely the cause for greater diversity (see Table 3). Carbon resources have been shown to impact the microbial diversity of environmental samples. High organic carbon content plays a significant role in structuring microbial communities by allowing invading species (bacteria) to easily become established (Zhou et al., 2002).

Understanding microbial diversity and the mechanisms which control community structure is important for the management of bioremediation processes (Zhou, et al., 2002). Microorganisms play an extremely important role in the global ecosystem by catalyzing many reactions important to life on earth. Contaminants such as explosive compounds can be incorporated into these reactions as an extension of normal microbial metabolism. The incorporation of contaminants into metabolic processes and overcoming the environmental constraints which govern these processes are major challenges in bioremediation efforts (Liu & Suflita, 1993).

Comparing the results between the two soil types indicates that Dorovan muck may be more viable for bioremediation efforts due to its higher microbial diversity. The abundant diversity present in Dorovan muck indicates that foreign bacteria are capable of becoming established, over time leading to increased microbial diversity. Bacteria which could be inoculated into Dorovan muck would likely become established due the abundant nutrients and carbon sources in this soil type. Conversely, Lakeland Soil is dominated by one phylogenetic family, Burkholderiaceae, and may not be amenable to inoculation of explosives degrading bacteria. Competition for nutrients has led to the overwhelming dominance of proteobacteria in Lakeland Soil (97% of diversity), and foreign bacteria are likely unable to compete with the microorganisms already present in this soil. As Lakeland Soil is the dominant soil type present

at Eglin AFB, and due to the lack of inherent RDX and HMX degradative function in this soil type, bioremediation should be viewed as unsuitable for explosives treatment at Eglin AFB.

Task 5. Obtain Permission from Eglin AFB for Field Study

A site visit to Eglin AFB was made on January 14, 2009 to meet with the Eglin Range Configuration Control Committee (RC3). The purpose of the RC3 meeting was to brief the committee on the scope of the phytoremediation project and to acquire clearance to plant vegetation on three plots near the open burn/open detonation (OB/OD) site. As a result of this meeting the use of phreatophytic tree species such as hybrid poplar was ruled out due to concerns with disturbing underlying UXO at the site. A site visit to Range C-62 was then conducted to finalize plans for field work with Three Rivers RC&D Council, Inc. (Milton, FL), the landscaping company which was chosen to perform the installation of vegetation. Planting Bahiagrass Pensacola in the form of sod offered a faster establishment period and a greater chance of survival over planting grass from seed.

Task 6. Plant Field Study Plots with Native Bahiagrass

Bahiagrass Pensacola sod was planted in three 0.4 acre plots: two "impacted" plots adjacent to the OB/OD site and one "control" plot located up-gradient of the site. The locations of the three plots are shown in Figure 56. Plot #1 is located east of the OB/OD site and was expected to contain the highest concentration of explosive compounds. Plot #2 is located south of the OB/OD site and was expected to be an area of lesser contamination. Plot #3, the control plot, is located to the north and was not expected to contain significant contamination. Planting took place in May 2009 and biannual soil and vegetation sampling is scheduled to occur through November 2010 to determine the effectiveness of Bahiagrass Pensacola on degrading energetic compounds.

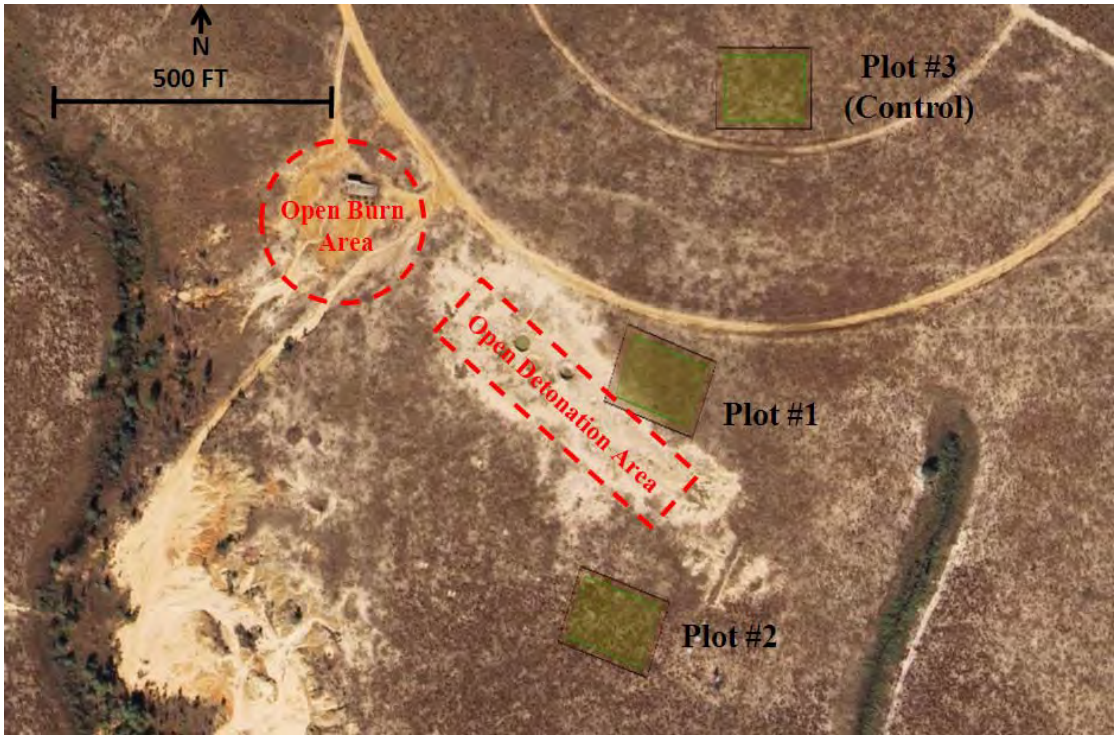


Figure 56. Area of phytoremediation field study at Eglin Air Force Base.

Planting of the Bahiagrass Pensacola sod at the site occurred on May 26-27, 2009. Three Rivers RC&D performed the site preparation, which consisted of removing the top layer of vegetation from each plot, and then installing the sod as shown in Figure 57 and Figure 58.

Figure 57. Installation of Bahiagrass Pensacola sod on May 26, 2009.

Figure 58. Installation of Bahiagrass Pensacola sod on May 26, 2009.

Initial watering to establish the sod was also provided by Three Rivers RC&D using a water truck. Unseasonably low precipitation occurred in the months of June and July 2009 at Eglin AFB, delaying successful establishment and vigorous growth of the sod until August and September 2009. Photographs of Plot #1 throughout the course of the field study are provided in Figure 59 through Figure 65.

Figure 59. Plot #1 on May 26, 2009.

Figure 60. Plot #1 on June 24, 2009.

Figure 61. Plot #1 on September 1, 2009.

Figure 62. Plot #1 on November 17, 2009.

Figure 63. Plot #1 on March 15, 2010.

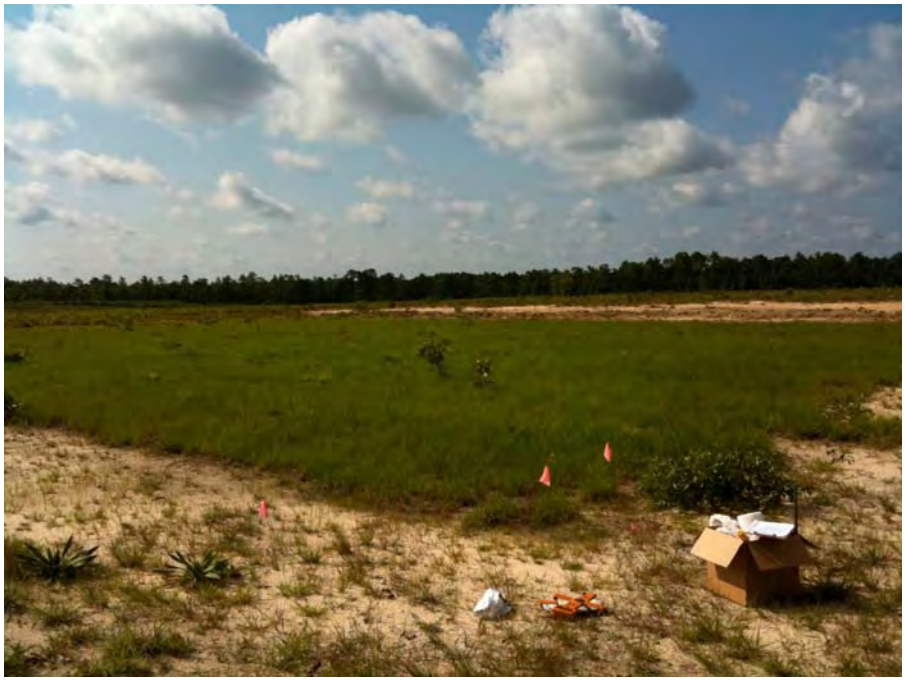


Figure 64. Plot #1 on May 22, 2010.

Figure 65. Plot #1 on November 13, 2010.

Task 7. Bi-Annual Sampling and Analysis of Soils from Field Study Plots

Results from Plots #2 and #3

568 discrete soil samples and 336 discrete plant samples from plots #2 and #3 were analyzed over the course of the four samplings. One detect was found in both plots #2 and #3 during the May 26-27, 2009 sampling and four detections were found in the plots during the November 13-14, 2010 sampling.

Results of Soil Analyzed by HPLC

The mean concentrations and standard deviations of each constituent detected by HLPC for all four samplings are given in Table 13 and Table 14. The means include non-detects as half of the limit of detection. Figure 66 and Figure 67 show the comparison explosive compound and metabolites mean concentrations for each sampling in the planted and unplanted regions of Plot #1.

Table 13. Mean and standard deviation from HPLC analysis of explosive compound and metabolite detections in Plot #1 during the May 26-27, 2009 and November 18-19, 2009 sampling. The analysis included non-detect samples as half the value of the limit of detection.

	LOD/2	May 26-27, 2009				November 18-19, 2009			
		Planted		Unplanted		Planted		Unplanted	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
HMX	0.02	0.13	0.60	0.46	2.49	0.07	0.34	0.06	0.09
RDX	0.08	0.23	0.54	4.08	24.37	0.30	1.71	0.27	0.70
TNT	0.10	0.11	0.01	0.11	0.02	0.11	0.04	0.10	0.01
TNB	0.05	0.06	0.03	0.08	0.15	0.06	0.05	0.05	0.00
2-ADNT	0.16	0.16	0.01	0.16	0.00	0.16	0.00	0.16	0.00
4-ADNT	0.21	0.21	0.00	0.21	0.01	0.21	0.00	0.21	0.00
2,4-DNT	0.09	0.44	3.02	0.49	2.36	0.10	0.01	0.12	0.19
2,6-DNT	0.15	0.17	0.17	0.17	0.13	0.15	0.00	0.15	0.00

Table 14. Mean and standard deviation from HPLC analysis of explosive compound and metabolite detections in Plot #1 during the May 24-25, 2010 and November 13-14, 2010 sampling. The analysis included non-detect samples as half the value of the limit of detection.

	LOD/2	May 24-25, 2010				November 13-14, 2010			
		Planted		Unplanted		Planted		Unplanted	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
HMX	0.02	0.13	0.63	0.12	0.46	0.03	0.06	0.09	0.16
RDX	0.08	0.13	0.24	0.08	0.00	0.09	0.05	0.10	0.12
TNT	0.10	0.10	0.01	0.10	0.00	0.10	0.00	0.10	0.00
TNB	0.05	0.05	0.00	0.05	0.00	0.05	0.00	0.05	0.00
2-ADNT	0.16	0.16	0.00	0.16	0.00	0.22	0.50	0.46	1.64
4-ADNT	0.21	0.21	0.00	0.21	0.00	0.21	0.00	0.21	0.00
2,4-DNT	0.09	0.11	0.16	0.09	0.00	0.12	0.24	0.23	0.73
2,6-DNT	0.15	0.15	0.01	0.15	0.00	0.15	0.00	0.15	0.00

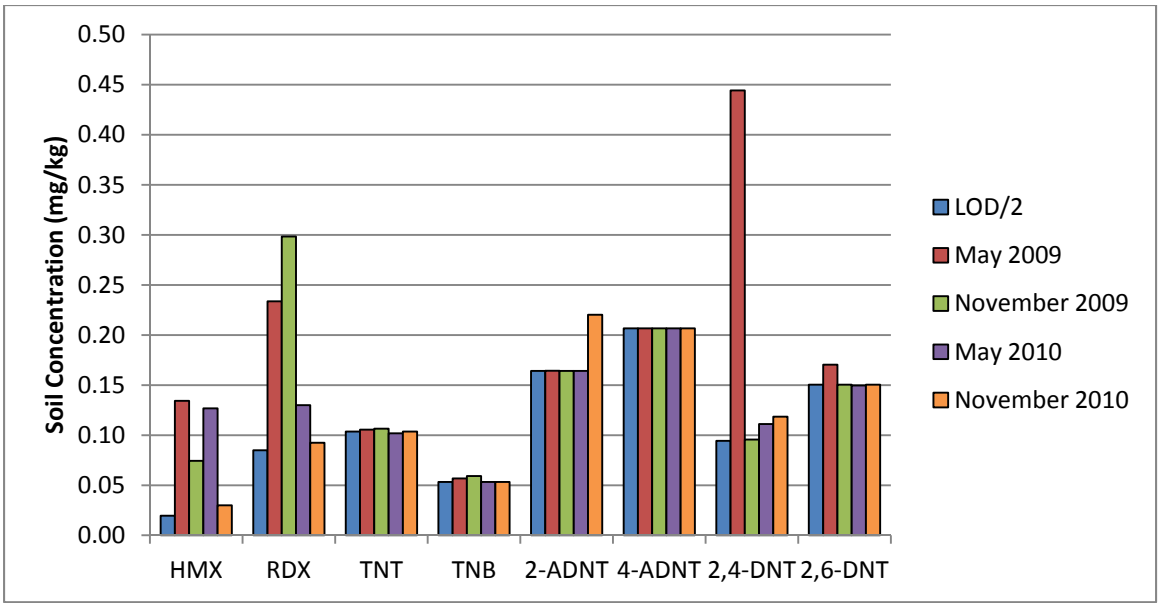


Figure 66. Mean concentration from HPLC analysis in the planted region of each constituent during the four samplings. Half the limit of detection serves as a reference for non-detect concentrations.

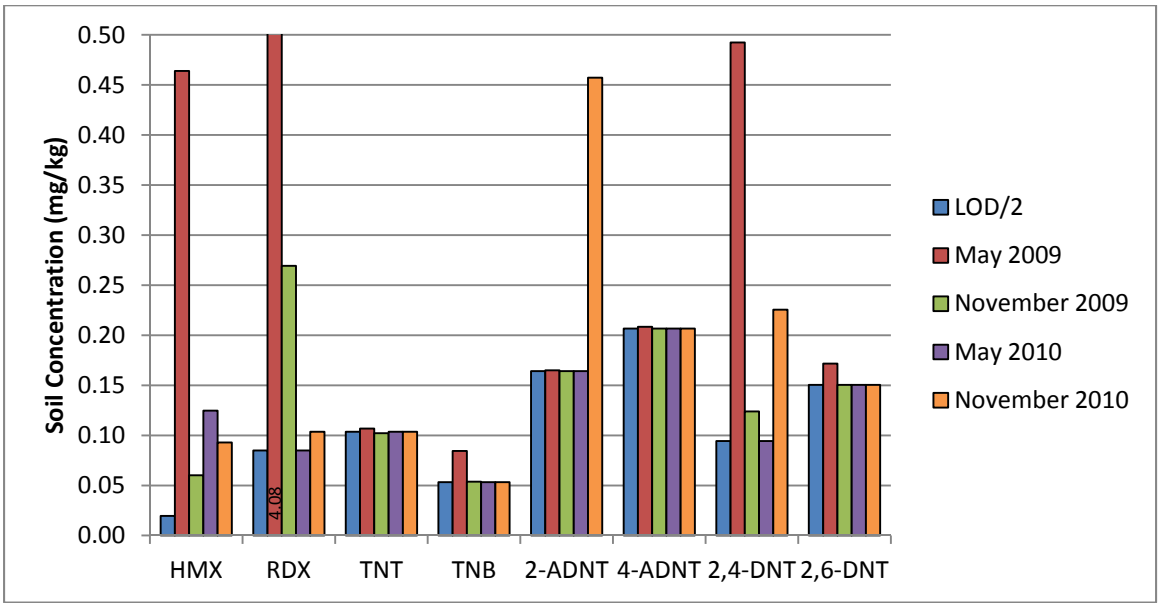


Figure 67. Mean concentration from HPLC analysis in the unplanted region of each constituent during the four samplings. Half the limit of detection serves as a reference for non-detect concentrations.

May 26-27, 2009 Sampling

The detections of explosive compounds in Plot #1 are shown in Figure 68 for the May 26-27, 2009 sampling. As seen in Figure 68, RDX and HMX were the two compounds most commonly detected and were also often found in the same discrete sample. Of the 100 discrete soil samples in the planted region of Plot #1, there were 18 detections of HMX ranging in concentration from 0.04 to 5.78 mg/kg, 30 detections of RDX ranging from 0.06 to 3.71 mg/kg, 3 detections of TNT ranging from 0.11 to 0.23 mg/kg, and 8 detections of TNT metabolites ranging from 0.10 to 29.91 mg/kg. Of the 40 discrete soil samples in the unplanted region, there were 12 detections of HMX ranging in concentration from 0.05 to 15.79 mg/kg, 13 detections of

RDX ranging from 0.07 to 154.30 mg/kg, 2 detections of TNT from 0.12 to 0.21 mg/kg, and 7 detections of TNT metabolites ranging from 0.19 to 15.02 mg/kg.

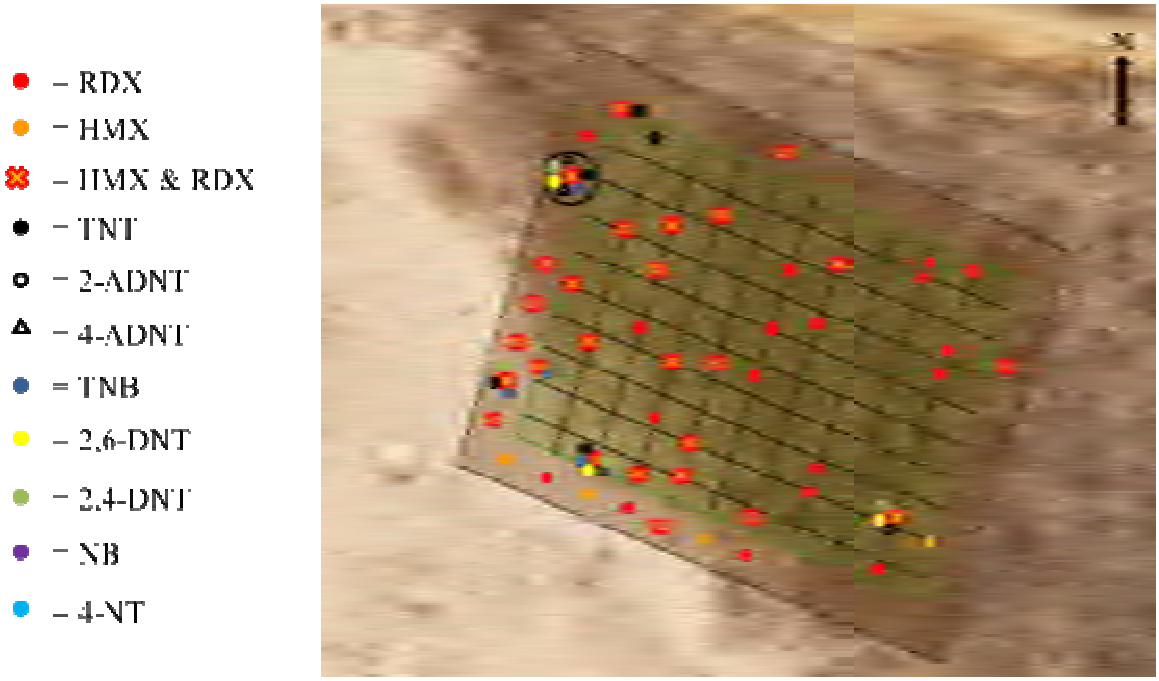


Figure 68. Plot #1 detections in soil for the May 26-27, 2009 sampling analyzed with HPLC. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

November 18-19, 2009 Sampling

The detections of explosive compounds in Plot #1 are shown in Figure 69 for the November 18-19, 2009 sampling. Again, the two most common compounds detected were RDX and HMX. Of the 100 discrete soil samples in the planted region of Plot #1, there were 22 detections of HMX ranging in concentration from 0.04 to 3.35 mg/kg, 33 detections of RDX ranging from 0.05 to 17.13 mg/kg, 3 detections of TNT ranging from 0.05 to 0.46 mg/kg, and 5 detections of TNT metabolites ranging from 0.04 to 0.50 mg/kg. Of the 40 discrete soil samples in the unplanted region, there were 14 detections of HMX ranging in concentration from 0.04 to 0.42 mg/kg, 19 detections of RDX ranging from 0.04 to 4.12 mg/kg, 1 detection of TNT at 0.04 mg/kg, and 3 detections of TNT metabolites ranging from 0.05 to 1.28 mg/kg.

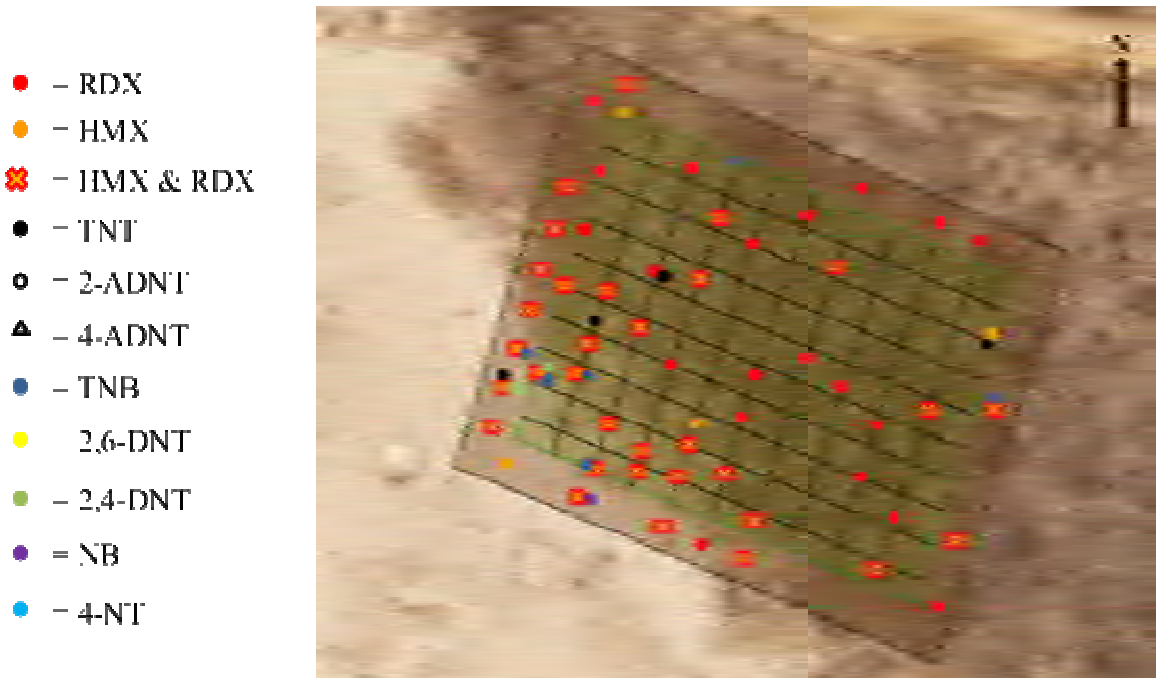


Figure 69. Plot #1 detections in soil for the November 18-19, 2009 sampling analyzed with HPLC. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

May 24-25, 2010 Sampling

The detections of explosive compounds in Plot #1 are shown in Figure 70 for the May 24-25, 2010 sampling. RDX and HMX were again the two most commonly detected compounds. From the 100 discrete soil samples taken, the planted region of Plot #1 had 18 detections of HMX ranging in concentration from 0.02 to 6.16 mg/kg, 13 detections of RDX ranging from 0.02 to 2.06 mg/kg, 7 detections of TNT ranging from 0.04 to 0.13 mg/kg, and 8 detections of TNT metabolites ranging from 0.07 to 1.71 mg/kg. Of the 40 discrete soil samples in the unplanted region, there were 15 detections of HMX ranging in concentration from 0.05 to 2.97 mg/kg. There were no detections of RDX, TNT, or TNT metabolites in the unplanted region during the May 24-25, 2010 sampling.

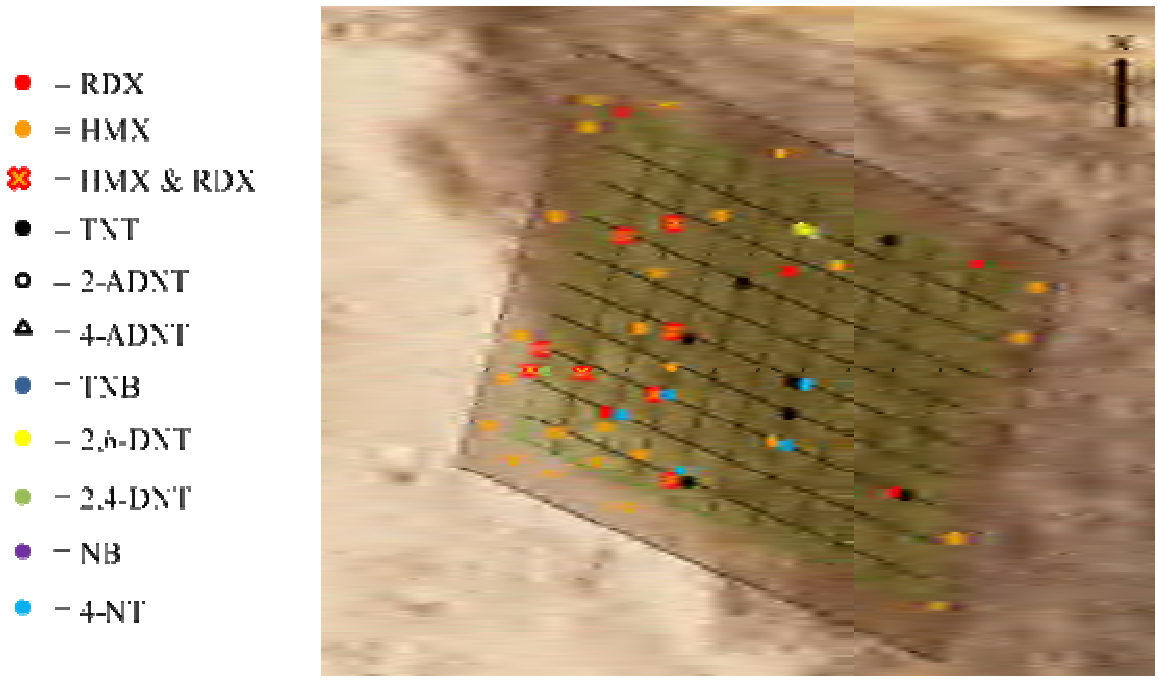


Figure 70. Plot #1 detections in soil for the May 24-25, 2010 sampling analyzed with HPLC. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

November 13-14, 2010 Sampling

The detections of explosive compounds in Plot #1 are shown in Figure 71 for the May 24-25, 2010 sampling. HMX was the most commonly detected compound. Of the 100 discrete soil samples in the planted region of Plot #1, there were 4 detections of HMX ranging in concentration from 0.05 to 0.45 mg/kg, 3 detections of RDX ranging from 0.11 to 0.50 mg/kg, and 3 detections of TNT metabolites ranging from 0.80 to 5.13 mg/kg. Of the 40 discrete soil samples in the unplanted region, there were 8 detections of HMX ranging in concentration from 0.23 to 0.69 mg/kg, 1 detection of RDX at 0.84 mg/kg, and 4 detections of TNT metabolites ranging from 0.73 to 10.50 mg/kg. There were no detections of TNT in the planted or unplanted region during the November 13-14, 2010 sampling.

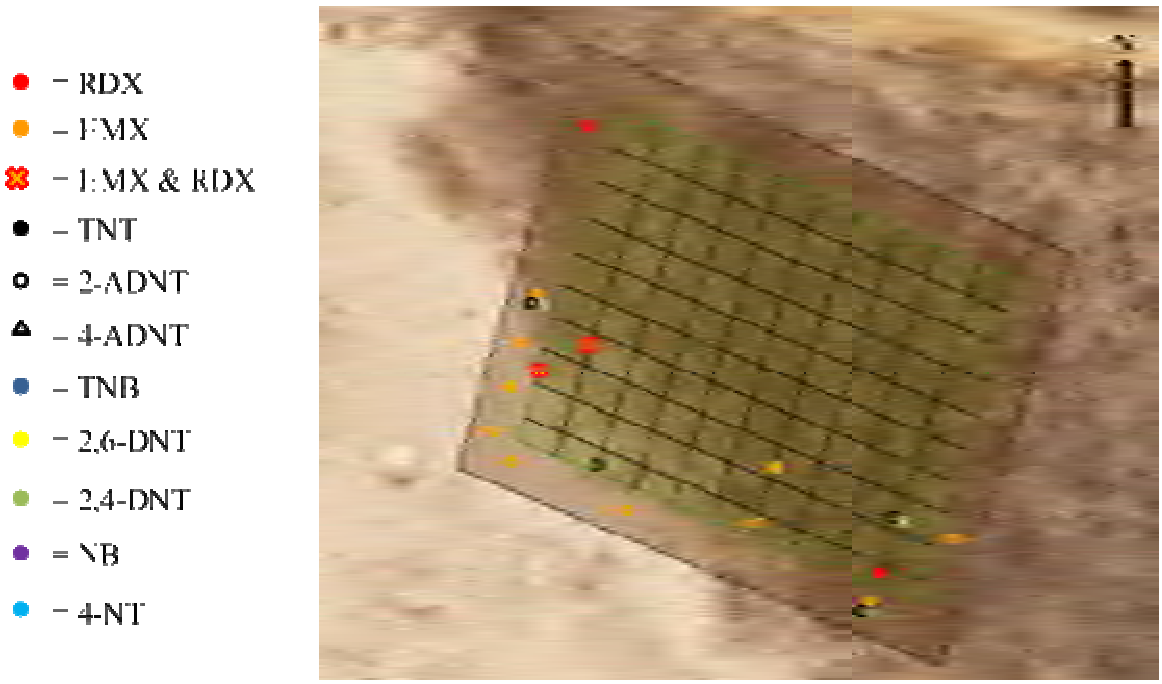


Figure 71. Plot #1 detections in soil for the November 13-14, 2010 sampling analyzed with HPLC. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

Discussion of Explosives in Soil Analyzed by HPLC

Figure 68 through Figure 71 show the detections of TNT, metabolites of TNT, RDX, or HMX in soil from analysis with HPLC. From these figures, it can be seen that the overall trend is toward fewer detections in both the planted and unplanted regions of Plot #1 for all compounds.

TNT plus Metabolites

Figure 72 and Figure 73 show the frequency histogram of TNT and TNT metabolite concentrations by HPLC analysis in the planted and unplanted regions, respectively, for the four samplings between May 26-27, 2009 and November 13-14, 2010. The most commonly detected TNT metabolites were 2-ADNT, 4-ADNT, 2,4-DNT, and 2,6-DNT. 2-ADNT and 4-ADNT have been shown to be formed from the aerobic reduction of TNT by plants (Hannink et al., 2002). 2,4-DNT and 2,6-DNT have also been observed as TNT metabolites (Schneider et al., 1996; Thompson et al., 1998).

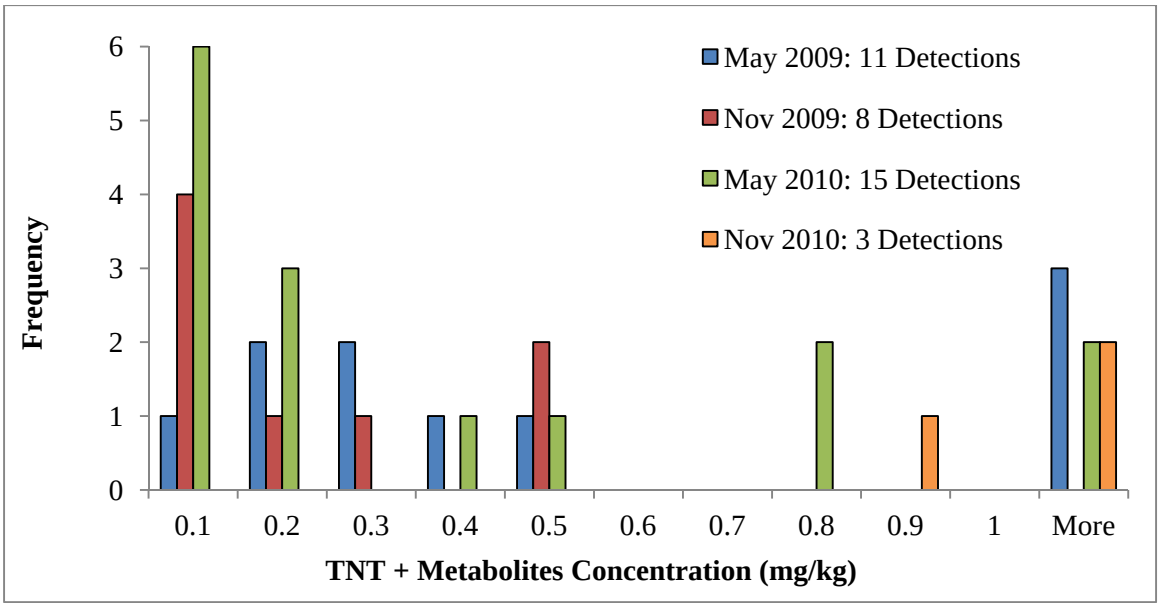


Figure 72. Frequency histogram of TNT plus metabolite soil concentrations found in the planted region of Plot #1 with HPLC through four successive samplings over one and a half years after planting.

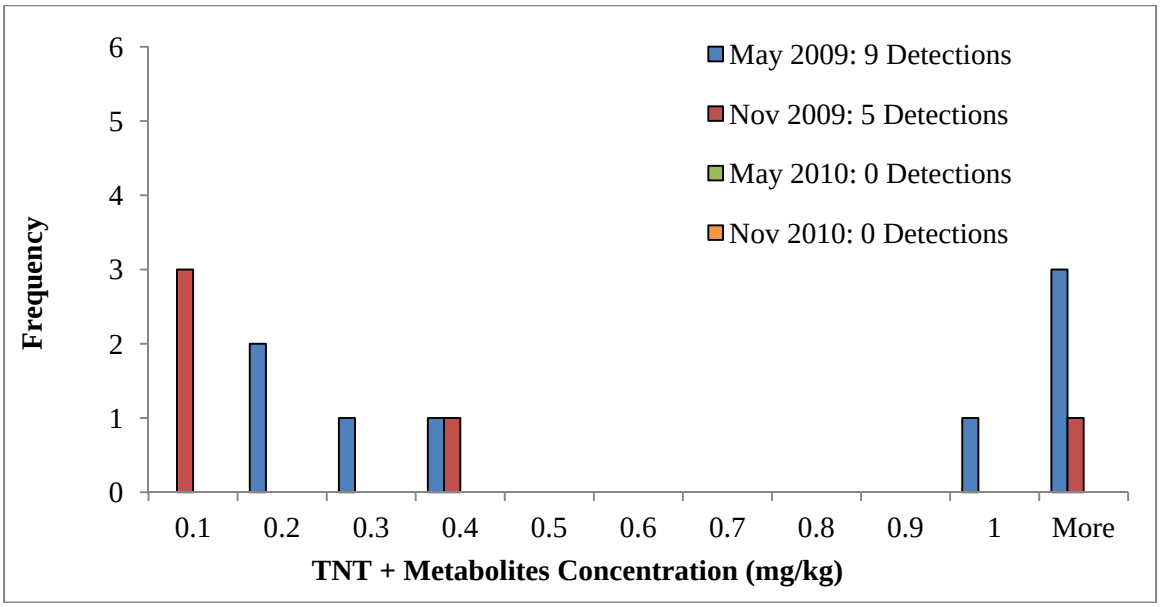


Figure 73. Frequency histogram of TNT plus metabolite soil concentrations found in the unplanted region of Plot #1 with HPLC through four successive samplings over one and a half years after planting.

Until the November 13-14, 2010 sampling, the TNT plus metabolite concentrations appear to be trending towards more detections at lower concentrations in the planted region of Plot #1 (see Figure 72). However, the November 13-14, 2010 results show only three detections, all of which are relatively greater in concentration compared to the majority of previous detections. In the unplanted region, the trend again appears to be greater detections at low concentrations over time (see Figure 73). There were no detections during the May 24-25, 2010 and November 13-14, 2010 samplings.

In Figure 66, which provides a better breakdown of TNT and each metabolite, it can be seen that the mean TNT concentration remains relatively constant in the planted region. The

figures depicting mean concentrations of the contaminants were constructed by including all non-detections as one-half the limit of detection. Due to the high number of non-detects and the relative greater concentration found in the samples where explosives were detected, the standard deviation of the means are very high as seen in Table 13 through Table 15. Even with the high standard deviations, the mean concentrations are still useful in the comparison of the mean concentration to half of the limit of detection (i.e. if the mean concentration of the contaminant is equal to half the limit of detection, little or no detections were found of significant concentration in the given sampling). Figure 66 depicts a high mean concentration of 2,4-DNT in the initial May 26-27, 2009 sampling, the November 13-14, 2010 sampling showed little to no presence of TNT metabolites, and the May 24-25, 2010 and November 13-14, 2010 samplings showed modest increases in the mean concentrations of 2,4-DNT and 2-ADNT. Figure 67 shows similar trends in the unplanted region: the mean TNT concentration remains relatively constant, a large mean concentration of 2,4-DNT is found in the initial sampling, and the mean metabolite concentrations increase in the final sampling.

These results may suggest the microbial communities in the soil are successfully degrading the compound since the TNT concentrations are remaining relatively constant throughout the study in both the planted and unplanted regions and the TNT metabolite concentrations are increasing. This result would confirm previous work accomplished in the laboratory, which showed 88% to 89% reduction in TNT concentrations over 28 days and 93% to 97% reduction over 56 days in unplanted Lakeland soil (Anderson, 2010). It is unclear if the implementation of phytoremediation is enhancing this process because rates could not be determined for the planted versus unplanted regions of Plot #1 due to the high number of samples below the limit of detection. This would greatly sway any mean, median, or parametric statistical analysis performed.

Although phytoremediation may not be enhancing the degradation or transformation of TNT, it appears that the organic carbon associated with plant roots and sod may be slowing the migration of TNT and metabolites. The mobility of TNT should decrease in the presence of organic carbon due to its affinity to partition to it as shown by the partition coefficients presented in Table 1. The decrease in mobility is shown through the comparison of detections of the planted and unplanted regions of the May 24-25, 2010 and November 13-14, 2010 samplings, exhibited in Figure 72 and Figure 73. Though the number of detections cannot be compared directly because of the differing frequency at which the samples were taken from each region (100 samples from the planted and 40 samples from the unplanted), it is clear that the fraction of detections to overall samples differ greatly in the planted and unplanted regions during the May 24-25, 2010 and November 13-14, 2010 samplings. The figures show that in the planted region sampled in May 24-25, 2010, 15% of the samples were above the limit of detection, whereas in the unplanted region, none of the samples were above the limit of detection. This was again seen in the November 13-14, 2010 sampling. In the planted region, 3% of the samples were above the limit of detection whereas in the unplanted region, none of the samples were above the limit of detection.

RDX

Figure 74 and Figure 75 show the frequency histogram of RDX concentrations by HPLC analysis in the planted and unplanted regions, respectively, for the four samplings between May 26-27, 2009 and November 13-14, 2010. In the planted region, the trend between the May 26-27, 2009 and November 18-19, 2009 was a greater number of detections at lower concentrations. Between November 18-19, 2009 and May 24-25, 2010 the number of detections in the planted

region decreased from 33 detections to 13 detections and finally to 3 detections in November 13-14, 2010 (see Figure 74). In the unplanted region of Plot #1, the same trend is followed. There were a greater number of detections at lower concentrations between May 26-27, 2009 and November 18-19, 2009. Between November 18-19, 2009 and May 24-25, 2010 the number of detections decreased from 19 to zero detections and the final sampling in November 13-14, 2010 resulted in only 1 detect. This trend is also seen in the mean RDX soil concentrations in the planted and unplanted regions shown in Figure 66 and Figure 67, respectively.

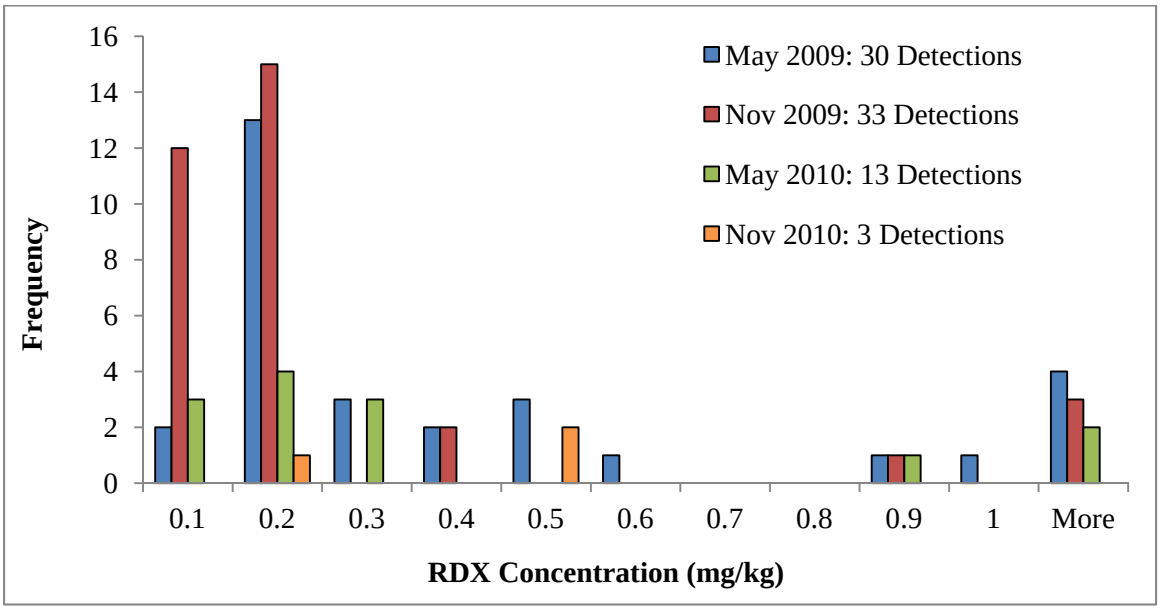


Figure 74. Frequency histogram of RDX soil concentrations found in the planted region of Plot #1 with HPLC through four successive samplings over one and a half years after planting.

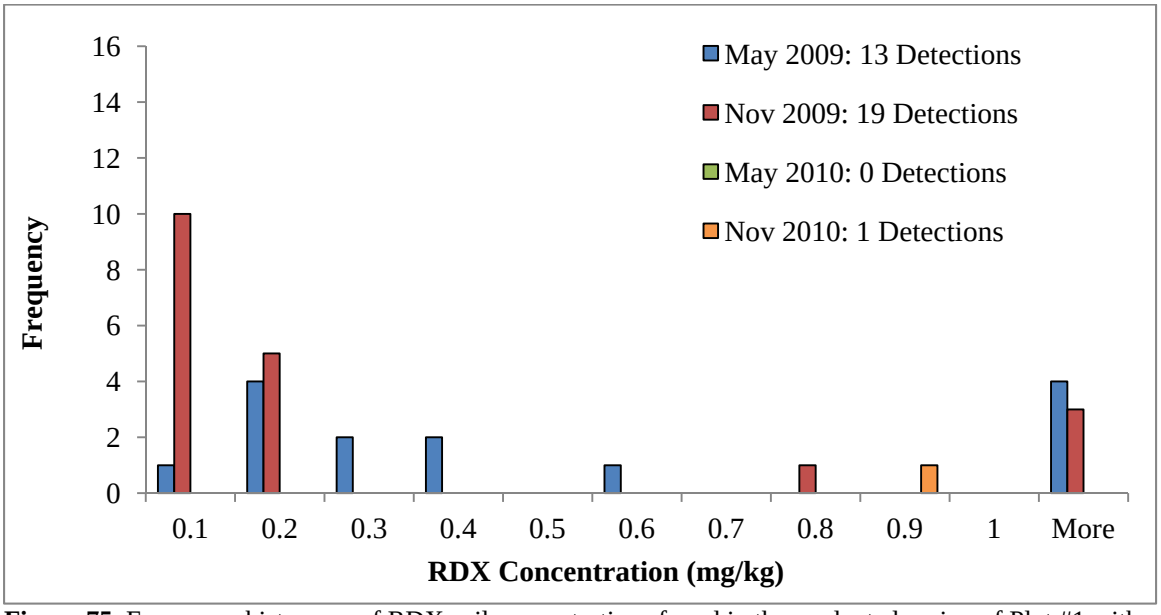


Figure 75. Frequency histogram of RDX soil concentrations found in the unplanted region of Plot #1 with HPLC through four successive samplings over one and a half years after planting.

A general linear model was applied to the data in order to determine if plant type (planted or unplanted), time (sampling date), or a combination of the two had a statistically significant effect on RDX concentration in the soil of Plot #1. Non-detections were not included because no parametric test can be performed due to the lack of normality in the distribution of the data as seen in Figure 76 and Figure 77. The results of the model are shown in Figure 78. The results show that time was statistically significant (P-value < 0.001) in the reduction of RDX concentrations in the soil while the plant type (unplanted or planted) had no significance.

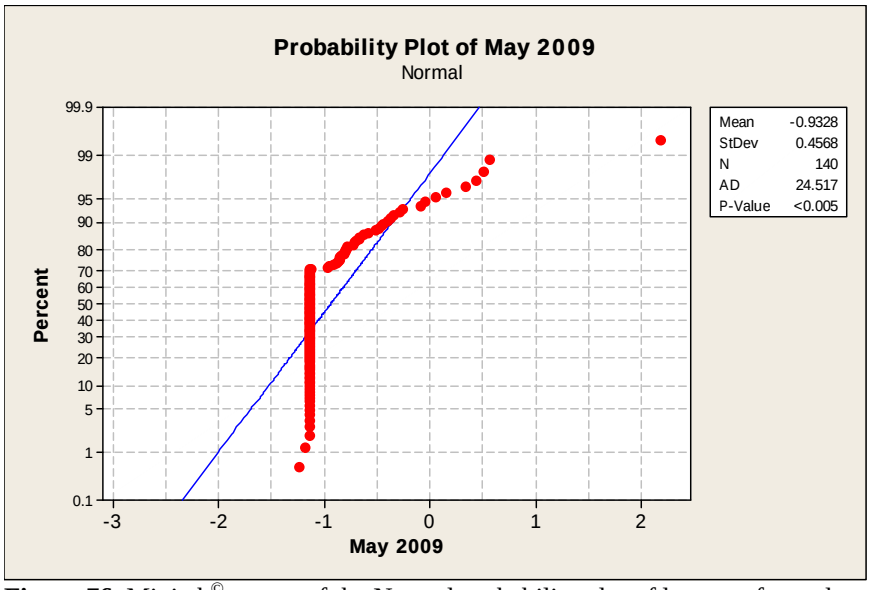


Figure 76. Minitab© output of the Normal probability plot of log transformed RDX soil concentrations from the May 26-27, 2009 sampling analyzed using HPLC.

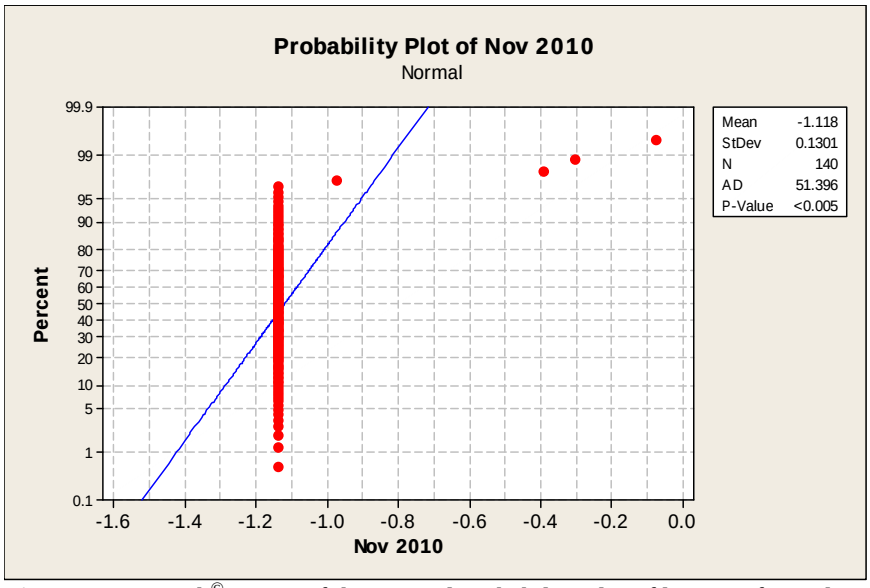


Figure 77. Minitab© output of the Normal probability plot of log transformed RDX soil concentrations from the November 13-14, 2010 sampling analyzed using HPLC.

General Linear Model: Log(RDX Conc) versus Plant Type, Time

```
Factor       Type   Levels  Values
Plant Type   fixed       2  0, 1
Time         fixed       4  1, 2, 3, 4

Analysis of Variance for Log(RDX Conc), using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS     F      P
Plant Type         1   0.0136   0.0331  0.0331  0.10  0.755
Time               3   5.4930   6.5429  2.1810  6.48  0.000
Plant Type*Time    3   1.3041   1.3041  0.4347  1.29  0.281
Error            109  36.7133  36.7133  0.3368
Total            116  43.5240

S = 0.580361   R-Sq = 15.65%   R-Sq(adj) = 10.23%

Unusual Observations for Log(RDX Conc)

      Log(RDX
Obs      Conc)       Fit    SE Fit  Residual  St Resid
 34   2.18838  -0.33822   0.16096   2.52659      4.53 R
 75   0.93317  -0.93296   0.09953   1.86613      3.26 R
 85   0.25903  -0.91039   0.12373   1.16942      2.06 R
 95   0.61520  -0.91039   0.12373   1.52559      2.69 R
113  -1.69897  -1.69897   0.58036   0.00000         * X
114  -0.39124  -0.55511   0.33507   0.16386      0.35 X
115  -0.30228  -0.55511   0.33507   0.25283      0.53 X
116  -0.97180  -0.55511   0.33507  -0.41669     -0.88 X
117  -0.07786  -0.07786   0.58036   0.00000         * X

R denotes an observation with a large standardized residual.
X denotes an observation whose X value gives it large leverage.
```

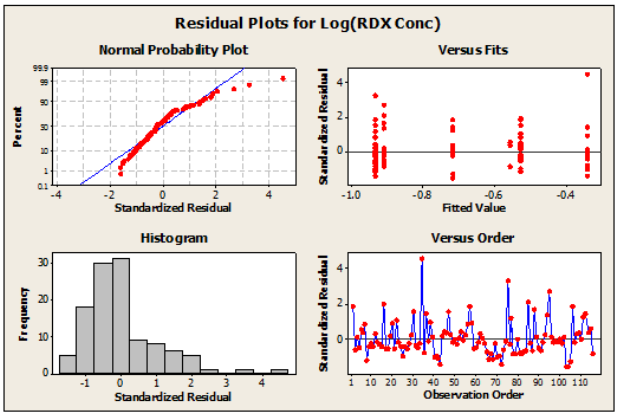


Figure 78. Minitab© output of the application of a General Linear Model on log-transformed RDX soil concentrations analyzed using HPLC.

Since the number of detections as well as concentrations is decreasing in both the planted and unplanted regions, it is believed that the RDX is migrating downward and into the groundwater faster than the Bahiagrass Pensacola plants can translocate the compound. This is conceivable given the solubility of RDX in water (Clausen et al., 2006) and its mobility in the environment (Dontsova, Yost, Simunek, Pennington, & Williford, 2006). Also, between the November 18-19, 2009 and May 24-25, 2010 sampling, Eglin AFB received its 5th wettest December on record and its 6th wettest January on record as shown by Figure 79 and Figure 80. These record rainfalls would have occurred while the Bahiagrass Pensacola was dormant, allowing for very little active uptake and phytoremediation to occur. In addition, Plot #1 was ignited on March 15, 2010 by the active use of the adjacent OB/OD site as shown in Figure 63. It is not believed that the grass fire had an effect on the reduction in RDX concentration in Plot

#1. The soil sample is taken 2 cm below the ground surface. It is unlikely that a grass fire could become hot enough to combust underlying explosive compounds.

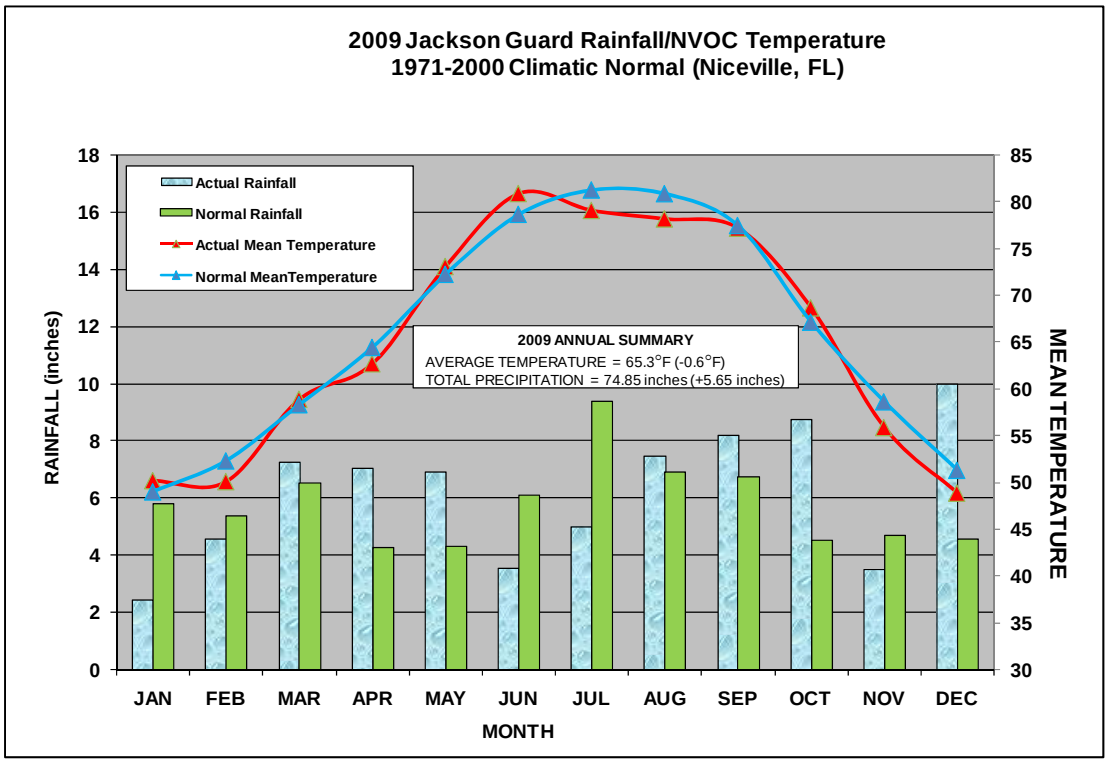


Figure 79. Summary of 2009 climate in Niceville, FL. Source: William "Sandy" Pizzolato

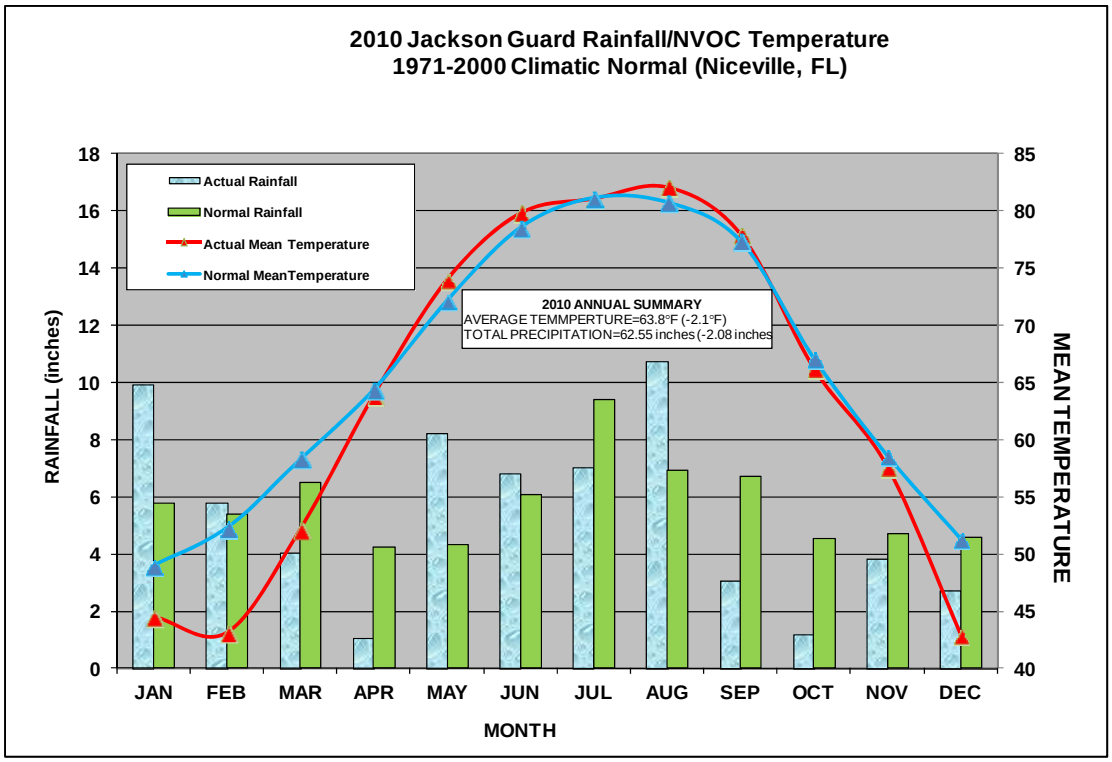


Figure 80. Summary of 2010 climate in Niceville, FL. Source: William "Sandy" Pizzolato

Additionally, the results of the field study are counter to those found in the laboratory microcosm study investigating the biodegradation of RDX in planted Eglin AFB soils. In the laboratory study, the RDX soil concentrations were reduced by 98.6% after 56 days in the planted Bahiagrass microcosm while the unplanted control saw no reductions. The difference in results is likely due to the laboratory microcosms being kept moist, yet unsaturated in order to keep the system aerobic. This led to little to no flow-through of water, which did not allow for the migration of RDX. This allowed for a much longer contact time between the roots and the soil and water and therefore led to greater treatment. In order to better represent the natural system, it is recommended that future laboratory studies incorporate flow-through. The mass of RDX in the flow-through water, the soil, and the plant material would better represent the processes occurring at Eglin AFB.

The reduction in mobility due to the presence of organic carbon via the sod and root layer was again seen for RDX. The mobility of RDX should decrease in the presence of organic carbon due to its affinity to partition to it, although not to the degree of TNT, as shown by the partition coefficients presented in Table 1. The decrease in mobility is shown through the comparison of detections of the planted and unplanted regions of the May 24-25, 2010 and November 13-14, 2010 samplings, exhibited in Figure 74 and Figure 75. Though the number of detections cannot be compared directly because of the differing frequency at which the samples were taken from each region (100 samples from the planted and 40 samples from the unplanted), it is clear that the fraction of detections to overall samples differ in the planted and unplanted regions during the May 24-25, 2010 and November 13-14, 2010 samplings. The figures show that in the planted region sampled in May 24-25, 2010 13% of the samples were above the limit of detection, whereas in the unplanted region, none of the samples were above the limit of detection. This was again seen in the November 13-14, 2010 sampling. However, there were fewer detections in both the planted and unplanted region, so the comparison is not as drastic. In the planted region, 3% of the samples were above the limit of detection whereas in the unplanted region, approximately 2% of the samples were above the limit of detection.

HMX

Figure 66 and Figure 67 show the mean HMX soil concentrations by HPLC analysis in the planted and unplanted regions, respectively, for the four samplings between May 26-27, 2009 and November 13-14, 2010. The figures show no discernible trend in the planted or the unplanted regions of Plot #1 from the mean concentrations of HMX in soil. The mean concentrations oscillate over the course of the four samplings.

Figure 81 and Figure 82 are histograms showing the distribution of HMX concentrations in soil in the planted and unplanted regions of Plot #1. In the planted region there appears to be a trend of greater detections at lower concentrations between the May 26-27, 2009 and November 18-19, 2009 samplings (see Figure 81). Between the May 24-25, 2010 and November 13-14, 2010 samplings, there are fewer detections. The detections are at lower concentrations when compared to the initial sampling. In the unplanted region, the trend of greater detections at lower concentrations extends from May 26-27, 2009 to May 24-25, 2010. The November 13-14, 2010 results are distributed differently than the other three samplings. Unlike the other three samplings, the November 13-14, 2010 had no detections less than 0.2 mg/kg (see Figure 82).

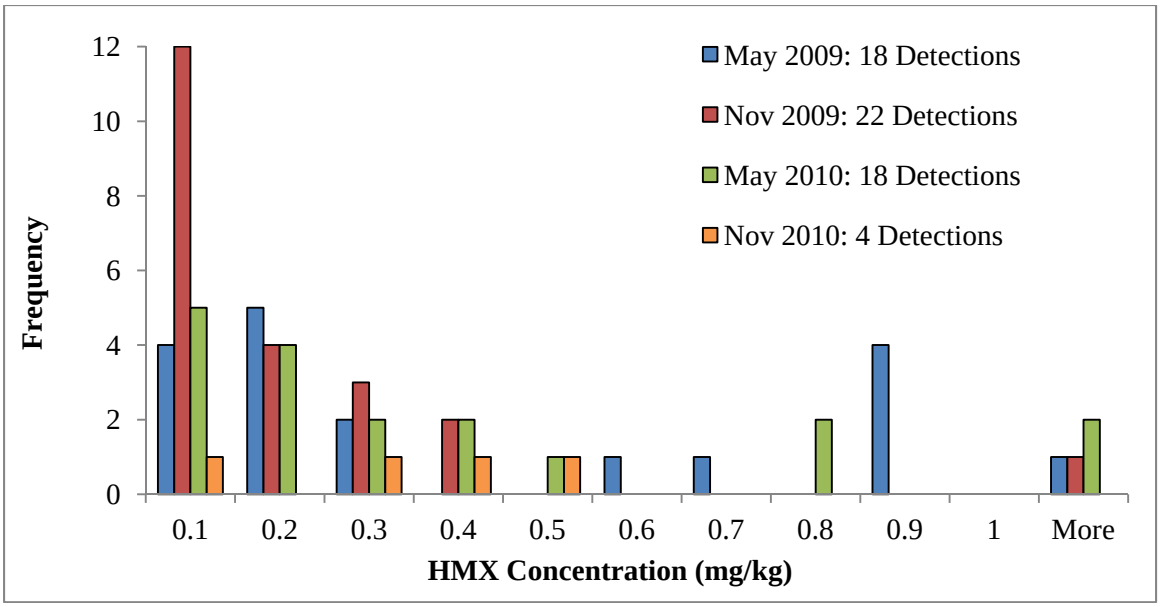


Figure 81. Frequency histogram of HMX soil concentrations found in the planted region of Plot #1 with HPLC through four successive samplings over one and a half years after planting.

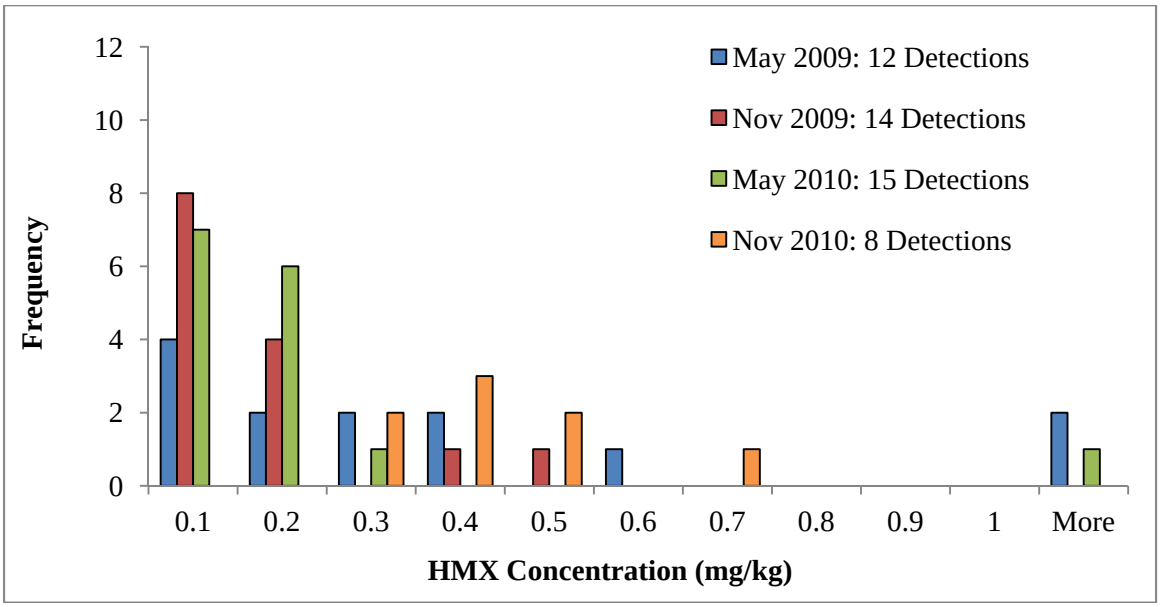


Figure 82. Frequency histogram of HMX soil concentrations found in the unplanted region of Plot #1 with HPLC through four successive samplings over one and a half years after planting.

A general linear model was applied to the data in order to determine if plant type (planted or unplanted), time (sampling date), or a combination of the two had a statistically significant effect on HMX concentration in the soil of Plot #1. Non-detections were not included because no parametric test can be performed due to the lack of normality in the distribution of the data as seen in Figure 83 and Figure 84. The results of the model are shown in Figure 85. The results show that time was statistically significant (P-value = 0.011) in the reduction of HMX concentrations in the soil while the plant type had no significance.

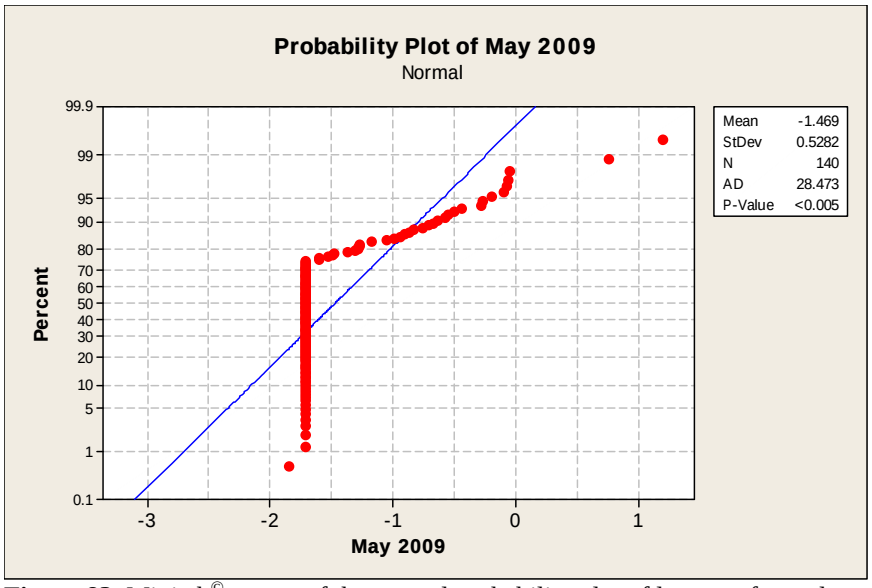


Figure 83. Minitab© output of the normal probability plot of log transformed HMX soil concentrations from the May 26-27, 2009 sampling analyzed using HPLC.

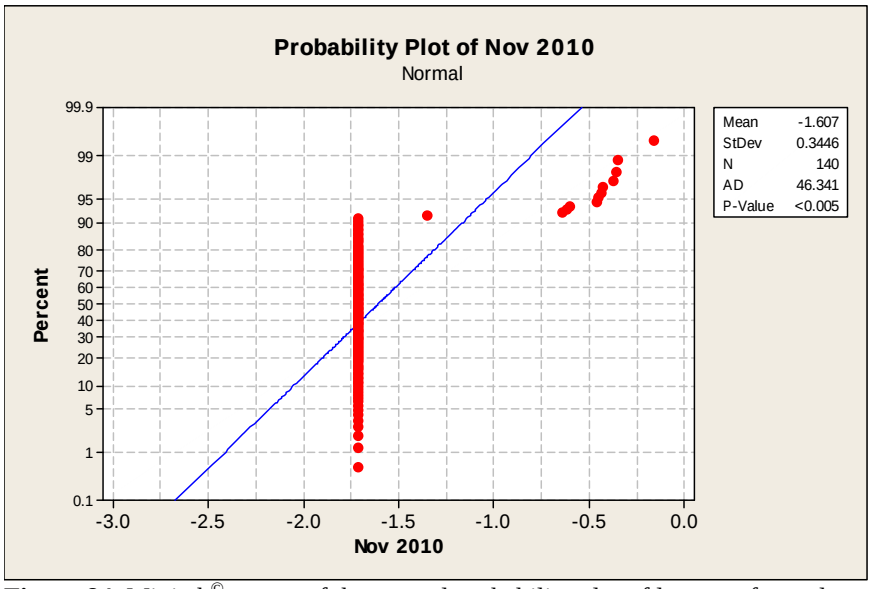


Figure 84. Minitab© output of the normal probability plot of log transformed HMX soil concentrations from the November 13-14, 2010 sampling analyzed using HPLC.

General Linear Model: Log(HMX Conc) versus Plant Type, Time

```
Factor       Type   Levels  Values
Plant Type   fixed       2  0, 1
Time         fixed       4  1, 2, 3, 4

Analysis of Variance for Log(HMX Conc), using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS      F      P
Plant Type         1   0.0025   0.0012  0.0012   0.00  0.950
Time               3   4.2433   3.5280  1.1760   3.88  0.011
Plant Type*Time    3   0.4804   0.4804  0.1601   0.53  0.663
Error            117  35.4273  35.4273  0.3028
Total            124  40.1534

S = 0.550270   R-Sq = 11.77%   R-Sq(adj) = 6.49%

Unusual Observations for Log(HMX Conc)

      Log(HMX
Obs      Conc)       Fit    SE Fit  Residual  St Resid
  8   0.76176  -0.76567   0.11732   1.52743      2.84 R
 11  -1.84128  -0.76567   0.11732  -1.07561     -2.00 R
 27   1.19835  -0.78916   0.14707   1.98751      3.75 R
 61   0.26240  -1.09749   0.10590   1.35989      2.52 R
 81   0.78958  -0.68725   0.12970   1.47684      2.76 R
113   0.47205  -0.89512   0.14208   1.36717      2.57 R
114  -0.60071  -0.68040   0.27514   0.07969      0.17 X
115  -1.34677  -0.68040   0.27514  -0.66636     -1.40 X
116  -0.42660  -0.68040   0.27514   0.25380      0.53 X
117  -0.34753  -0.68040   0.27514   0.33287      0.70 X

R denotes an observation with a large standardized residual.
X denotes an observation whose X value gives it large leverage.
```

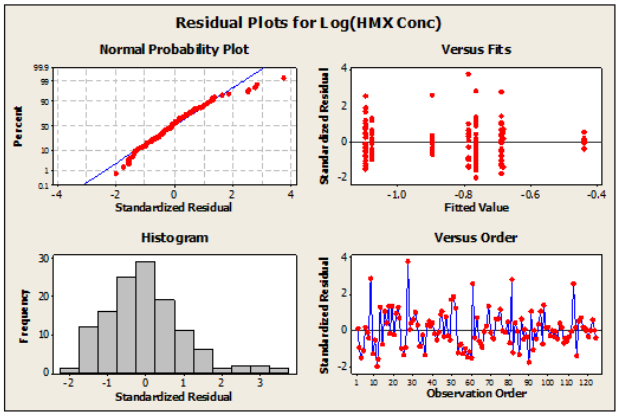


Figure 85. Minitab© output of the application of a General Linear Model on log-transformed HMX soil concentrations analyzed using HPLC.

While HMX is much more recalcitrant due to its poor solubility compared to TNT and RDX, it is somewhat mobile once solubilized and has been shown to accumulate in leaves (Hannink et al., 2002; Groom et al., 2002; Yoon et al., 2002; Yoon et al., 2006). However, the same trend was observed as with RDX. More detections of HMX were observed at lower concentration over the study in both the planted and unplanted regions of Plot #1. Therefore, phytoremediation using Bahiagrass Pensacola must not be having a substantial effect on the reduction of HMX concentrations in the soil. The evidence points to HMX migrating downward in profile and diluting the soil concentration.

The organic carbon found in the sod and root zone did not appear to have an effect on the mobility of HMX. This is surprising given that the partition coefficients, found in Table 1, are of

the same order of magnitude as those for RDX, which did appear to be hindered by the presence of organic carbon. The differing behavior may be caused by the decreased mobility of HMX due to its decreased solubility as compared to TNT and RDX.

Results of Soil Analyzed by LC/MS

The mean concentrations and standard deviations of each constituent detected by LC/MS for all four samplings are given in Table 15. Figure 86 and Figure 87 show the comparison of explosive compound and metabolite mean concentrations for the May 24-25, 2010 and November 13-14, 2010 samplings in the planted and unplanted regions of Plot #1.

Table 15. Mean and standard deviation from LC/MS analysis of explosive compound and metabolite detections in Plot #1 during the May and November 13-14, 2010 sampling. The analysis included non-detect samples as half the value of the limit of detection.

	LOD/2	May 24-25, 2010				November 13-14, 2010			
		Planted		Unplanted		Planted		Unplanted	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
HMX	0.001	0.153	0.832	0.041	0.133	0.021	0.055	0.069	0.161
RDX	0.079	0.123	0.227	0.085	0.052	0.087	0.069	0.079	0.000
TNT	0.005	0.005	0.000	0.005	0.000	0.006	0.010	0.005	0.000
TNB	0.026	0.027	0.010	0.026	0.000	0.027	0.012	0.026	0.000
2-ADNT	0.005	0.007	0.007	0.005	0.002	0.007	0.011	0.007	0.008
4-ADNT	0.003	0.006	0.010	0.004	0.004	0.003	0.002	0.005	0.006
2,4-DNT	0.009	0.009	0.000	0.009	0.000	0.015	0.047	0.031	0.143
2,6-DNT	0.100	0.117	0.172	0.100	0.000	0.147	0.300	0.214	0.720

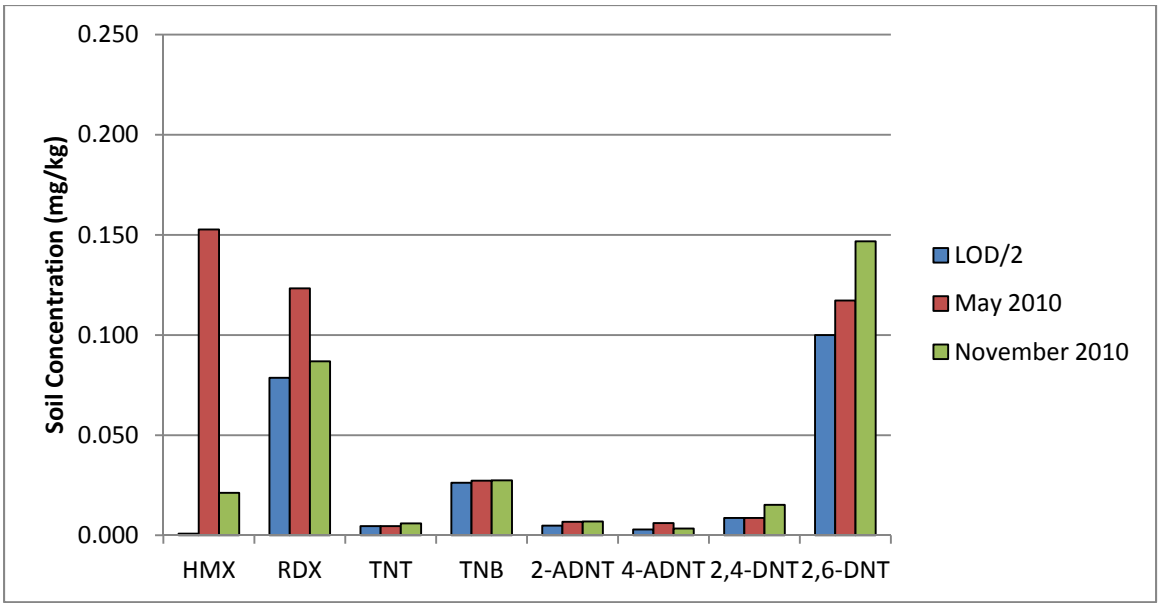


Figure 86. Mean concentration from LC/MS analysis in the planted region of each constituent during the May 24-25, 2010 and November 13-14, 2010 samplings. Half the limit of detection serves as a reference for non-detect concentrations.

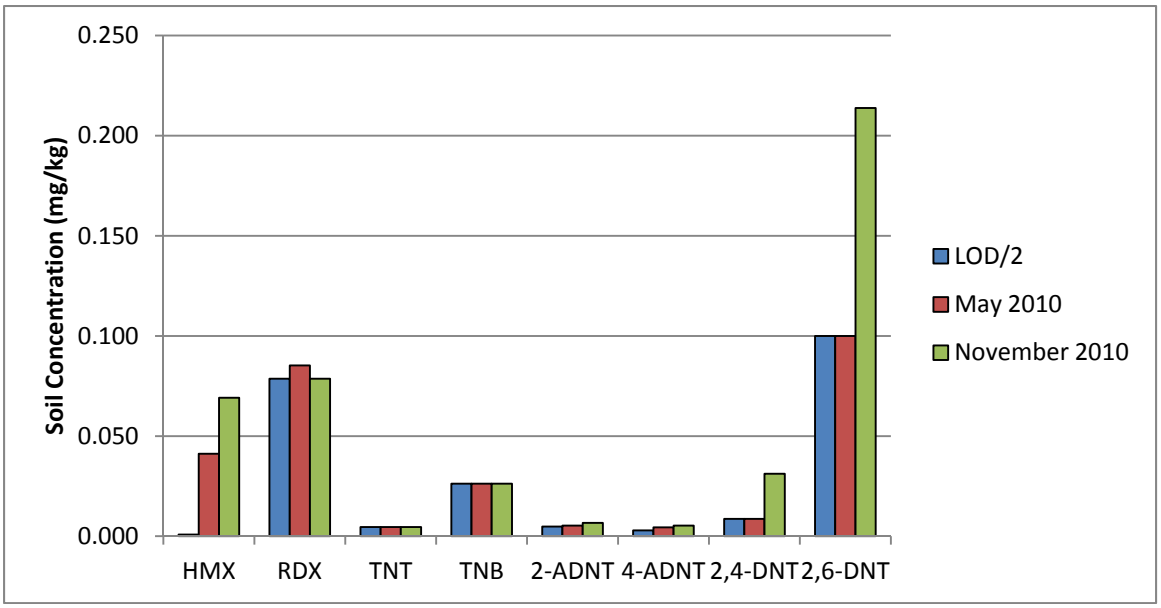


Figure 87. Mean concentration from LC/MS analysis in the unplanted region of each constituent during the May 24-25, 2010 and November 13-14, 2010 samplings. Half the limit of detection serves as a reference for non-detect concentrations.

May 24-25, 2010 Sampling

The detections of explosive compounds in Plot #1 are shown in Figure 88 for the May 24-25, 2010 sampling. HMX and RDX were the most commonly detected compounds. Of the 100 discrete soils samples in the planted region of Plot #1, there were 72 detections of HMX ranging in concentration from 0.002 to 8.161 mg/kg, 26 detections of RDX ranging from 0.03 to 1.87 mg/kg, and 34 detections of TNT metabolites (predominantly 2-ADNT; 4-ADNT) ranging from 0.003 to 1.821 mg/kg. Of the 40 discrete soil samples in the unplanted region, there were

35 detections of HMX ranging in concentration from 0.002 to 0.846 mg/kg, 12 detections of RDX ranging from 0.03 to 0.33 mg/kg, and 18 detections of TNT metabolites (exclusively 2-ADNT; 4-ADNT) ranging from 0.003 to 0.016 mg/kg. There were no detections of TNT in the planted or unplanted region during the May 24-25, 2010 sampling.

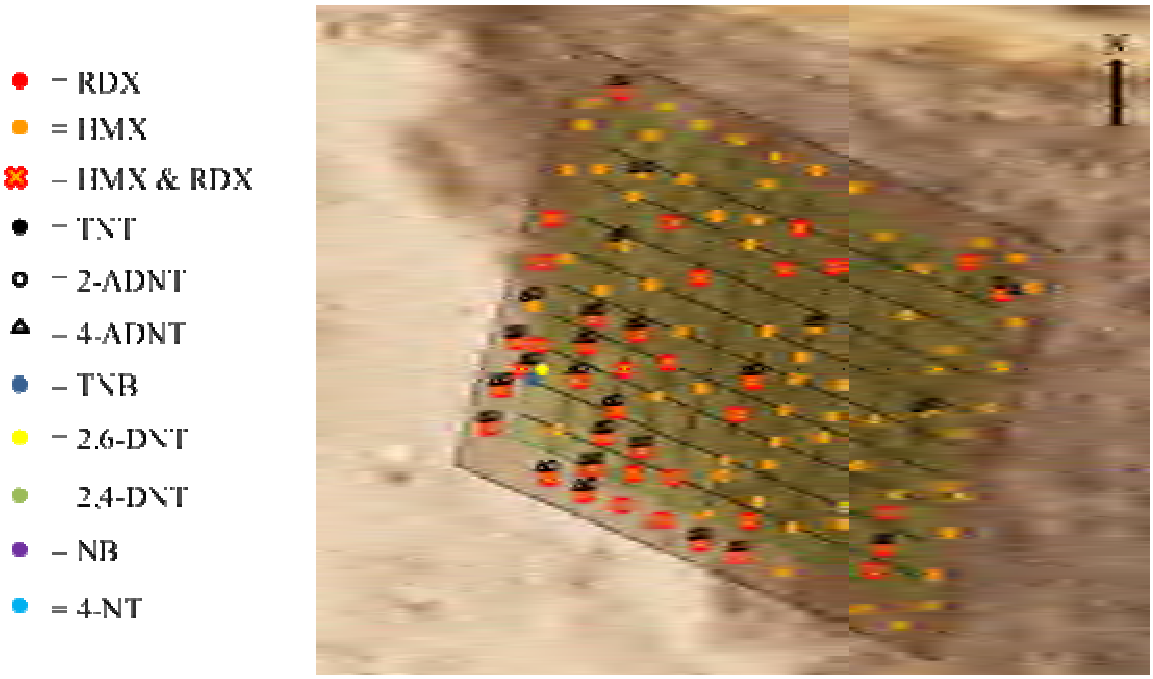


Figure 88. Plot #1 detections in soil for the May 24-25, 2010 sampling analyzed with LC/MS. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

November 13-14, 2010 Sampling

The detections of explosive compounds in Plot #1 are shown in Figure 89 for the November 13-14, 2010 sampling. HMX was the most commonly detected compound. Of the 100 discrete soil samples in the planted region of Plot #1, there were 54 detections of HMX ranging in concentration from 0.003 to 0.381 mg/kg, 3 detections of RDX ranging from 0.10 to 0.75 mg/kg, 2 detections of TNT ranging from 0.05 to 0.10 mg/kg, and 17 detections of TNT metabolites ranging from 0.006 to 2.405 mg/kg. Of the 40 discrete soil samples in the unplanted region, there were 27 detections of HMX ranging in concentration from 0.004 to 0.790 mg/kg and 13 detections of TNT metabolites ranging from 0.002 to 4.654 mg/kg. There were no detections of TNT or RDX in the unplanted region during the November 13-14, 2010 sampling.

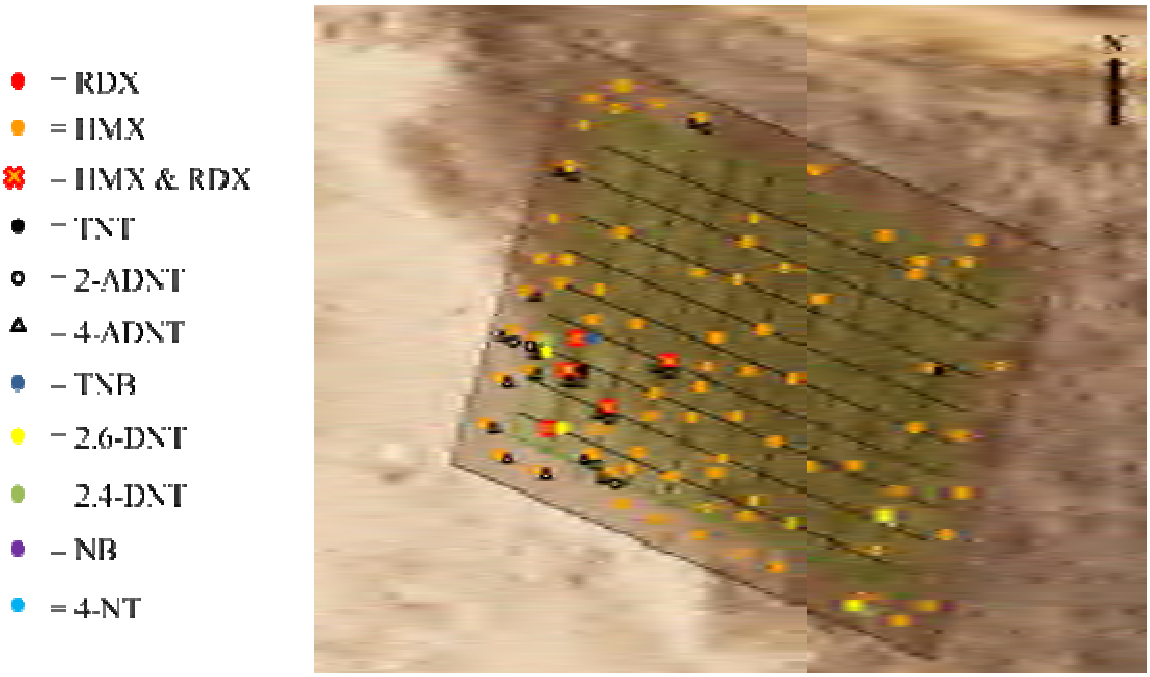


Figure 89. Plot #1 detections in soil for the November 13-14, 2010 sampling analyzed with LC/MS. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

Discussion of Explosives in Soil Analyzed by LC/MS

Figure 88 and Figure 89 show the detections of TNT, metabolites of TNT, RDX, or HMX in soil from analysis with LC/MS. From these figures, it can be seen that the general trend is toward fewer detections in both the planted and unplanted regions of Plot #1. The main purpose of the analysis with LC/MS was to reaffirm detections and concentrations of explosive compounds in the soil. Certainty in trends cannot be determined with only two samplings analyzed.

TNT plus Metabolites

Figure 90 and Figure 91 show the frequency histogram of TNT and TNT metabolite concentrations by LC/MS analysis in the planted and unplanted regions, respectively, for the samplings in May 24-25, 2010 and November 13-14, 2010. The two figures show a trend of an increase towards greater TNT plus metabolite concentrations. Figure 86 and Figure 87 also show that the mean TNT concentration remained approximately the same as the mean concentration of TNT metabolites increased in both the planted and unplanted regions of Plot #1. This was also the case with the HPLC results. Therefore, the same conclusion is reached: microbial communities in the soil are successfully transforming the TNT. It is again unclear if the implementation of Bahiagrass Pensacola for phytoremediation is having an effect on the rate of transformation because the process is occurring in both the planted and unplanted regions.

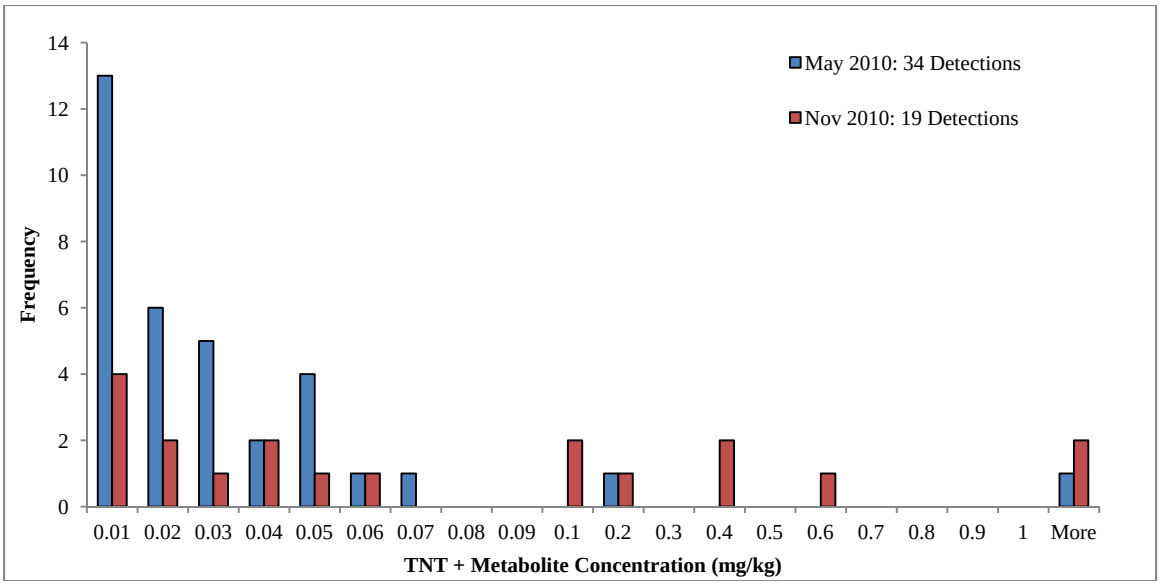


Figure 90. Frequency histogram of TNT plus metabolite soil concentrations found in the planted region of Plot #1 with LC/MS only through the May and November 13-14, 2010 samplings.

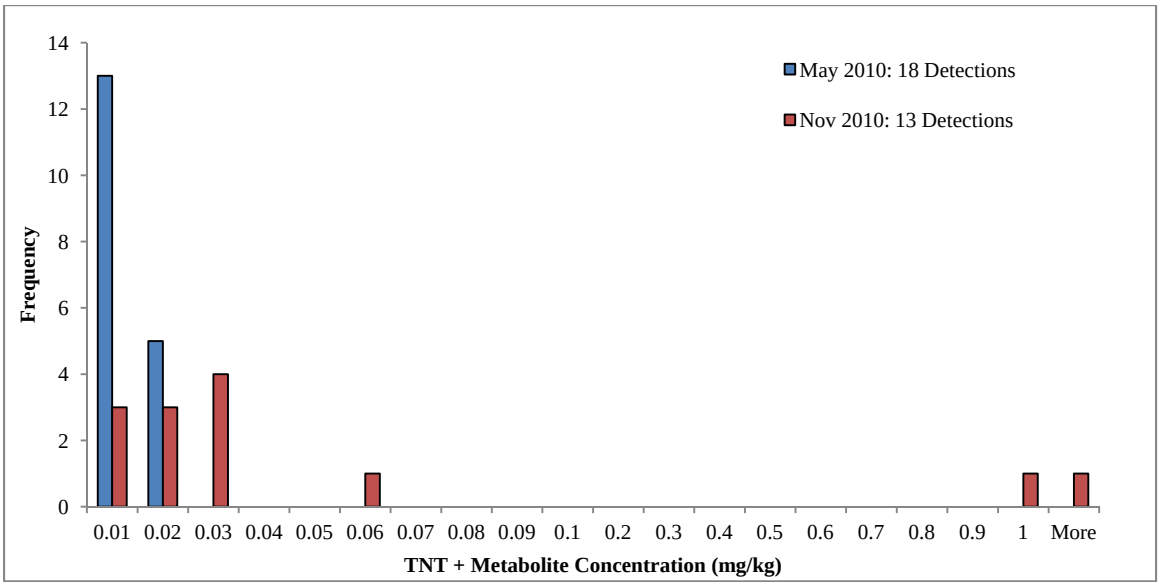


Figure 91. Frequency histogram of TNT plus metabolite soil concentrations found in the unplanted region of Plot #1 with LC/MS only through the May and November 13-14, 2010 samplings.

RDX

Figure 92 and Figure 93 show the frequency histogram of RDX concentrations by LC/MS analysis in the planted and unplanted regions, respectively, for the May 24-25, 2010 and November 13-14, 2010 samplings. As seen in both figures, there is no clear change in distribution between the two samplings. Fewer detections were observed in both the planted and unplanted regions of Plot #1 during the November 13-14, 2010 sampling compared to the May 24-25, 2010 sampling. This follows the same trend observed in the HPLC data indicating that the RDX is migrating downward in both the planted and unplanted regions.

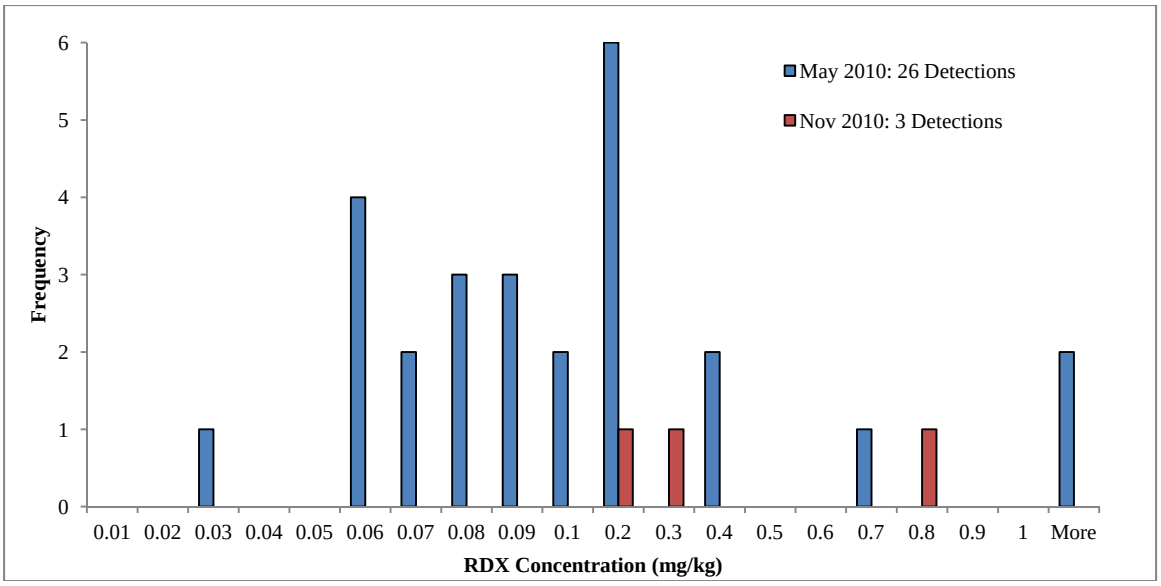


Figure 92. Frequency histogram of RDX soil concentrations found in the planted region of Plot #1 with LC/MS only through the May and November 13-14, 2010 samplings.

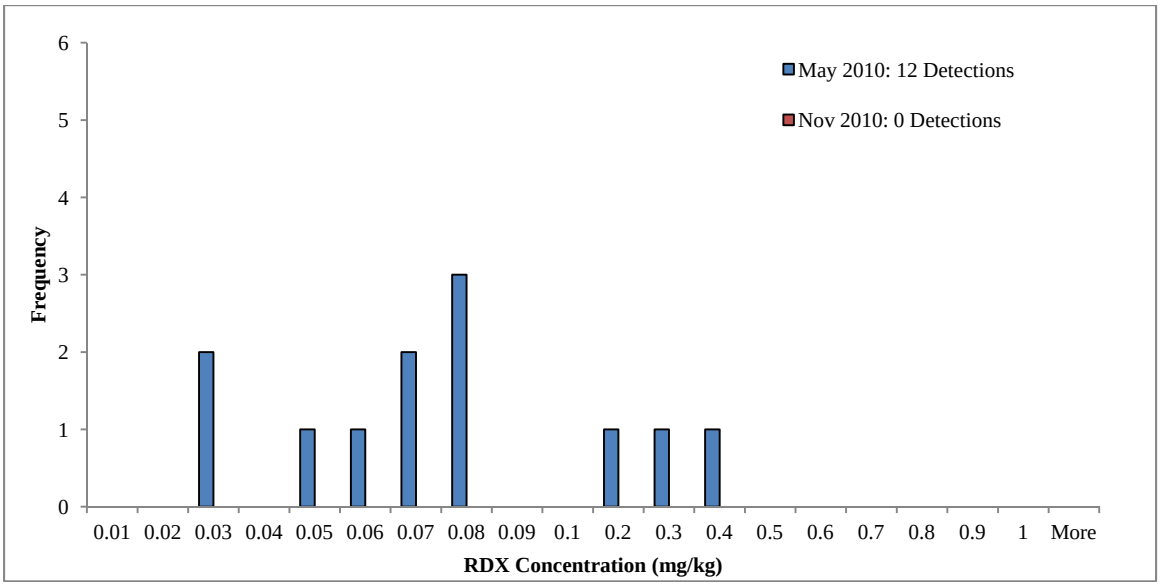


Figure 93. Frequency histogram of RDX soil concentrations found in the unplanted region of Plot #1 with LC/MS.

A general linear model was applied to the data in order to determine if plant type (planted or unplanted), time (sampling date), or a combination of the two had a statistically significant effect on RDX concentrations in the soil of Plot #1. Non-detections were not included because no parametric test can be performed due to the lack of normality in the distribution of the data as seen in Figure 94 and Figure 95. The results of the model are shown in Figure 96. The results show that neither time nor plant type was statistically significant in the reduction of RDX concentrations in soil according to the LC/MS data.

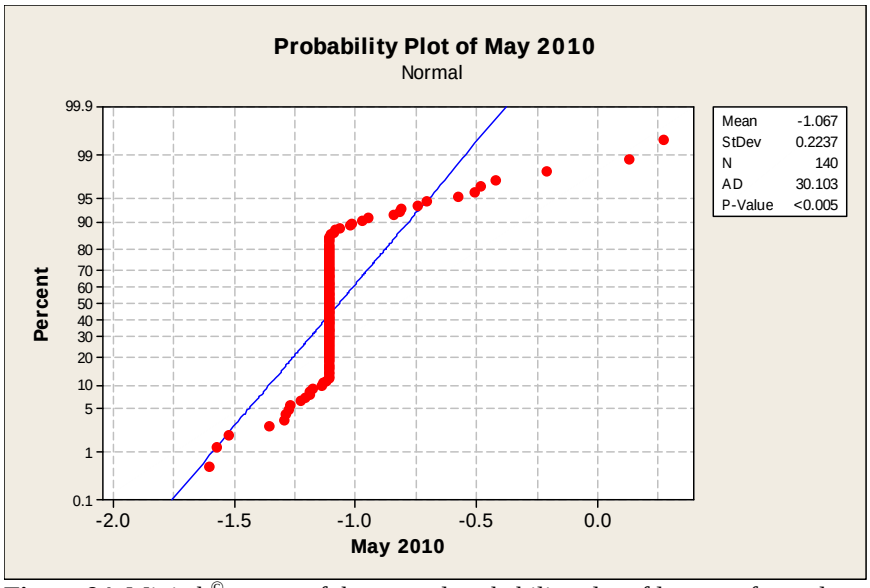


Figure 94. Minitab© output of the normal probability plot of log transformed RDX soil concentrations from the May 24-25, 2010 sampling analyzed using LC/MS.

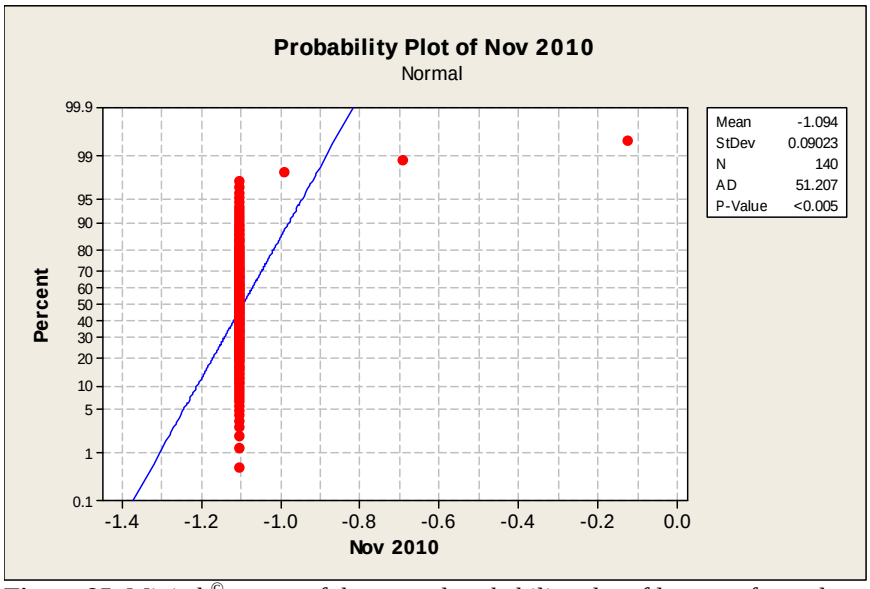


Figure 95. Minitab© output of the normal probability plot of log transformed RDX soil concentrations from the November 13-14, 2010 sampling analyzed using LC/MS.

General Linear Model: Log(RDX Conc) versus Plant Type, Time

```
Factor       Type   Levels  Values
Plant Type   fixed       2  0, 1
Time         fixed       2  1, 2

Analysis of Variance for Log(RDX Conc), using Adjusted SS for Tests

Source           DF  Seq SS  Adj SS  Adj MS     F      P
Plant Type        1  0.5202  0.1318  0.1318  0.79  0.381
Time              1  0.3262  0.2618  0.2618  1.56  0.219
Plant Type*Time   1  0.0008  0.0008  0.0008  0.00  0.947
Error            38  6.3708  6.3708  0.1677
Total            41  7.2180

S = 0.409453   R-Sq = 11.74%   R-Sq(adj) = 4.77%

Unusual Observations for Log(RDX Conc)

      Log(RDX
Obs      Conc)      Fit    SE Fit  Residual  St Resid
 13   0.27255  -0.89295   0.08030   1.16550      2.90 R
 23   0.12957  -0.89295   0.08030   1.02253      2.55 R
 39  -0.98898  -0.60096   0.23640  -0.38802     -1.16 X
 40  -0.69135  -0.60096   0.23640  -0.09039     -0.27 X
 41  -0.12255  -0.60096   0.23640   0.47841      1.43 X
 42  -0.80327  -0.80327   0.40945  -0.00000         * X

R denotes an observation with a large standardized residual.
X denotes an observation whose X value gives it large leverage.
```

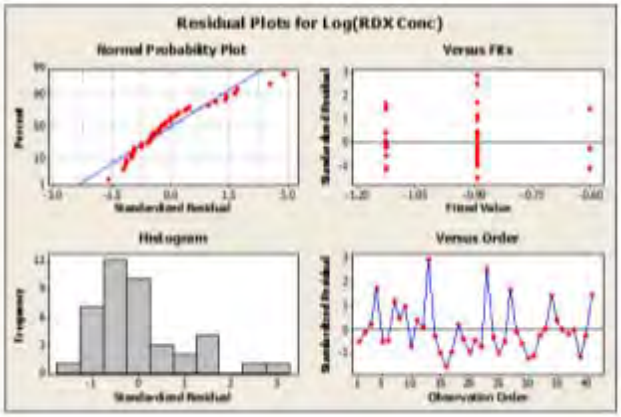


Figure 96. Minitab© output of the application of a General Linear Model on log-transformed RDX soil concentrations analyzed using LC/MS.

From the histograms shown in Figure 92 and Figure 93, it is believed that the RDX is migrating downward into the groundwater faster than Bahiagrass Pensacola is able to uptake the compound. This same conclusion was reached from the analysis of the HPLC data.

HMX

Figure 97 and Figure 98 show the frequency histograms of HMX concentrations by LC/MS analysis in the planted and unplanted regions, respectively, for the May 24-25, 2010 and November 13-14, 2010 samplings. In the planted region, there appears to be a trend towards greater detections at lower concentrations when comparing the May 24-25, 2010 and November 13-14, 2010 samplings. In the unplanted region, the trend of greater detections at lower concentrations is not apparent. In fact, there appears to be more detections of higher concentrations. The reduction of HMX concentrations in the planted region and the increase of

HMX concentrations in the unplanted region are also shown by the mean HMX soil concentrations in Figure 86 and Figure 87, respectively.

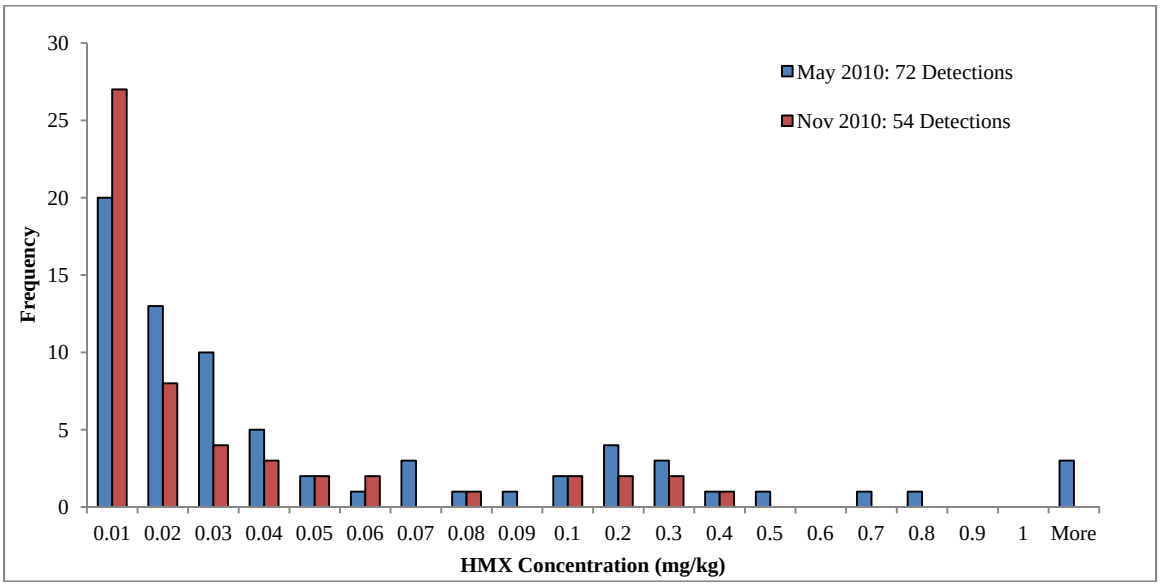


Figure 97. Frequency histogram of HMX soil concentrations found in the planted region of Plot #1 with LC/MS only through the May and November 13-14, 2010 samplings.

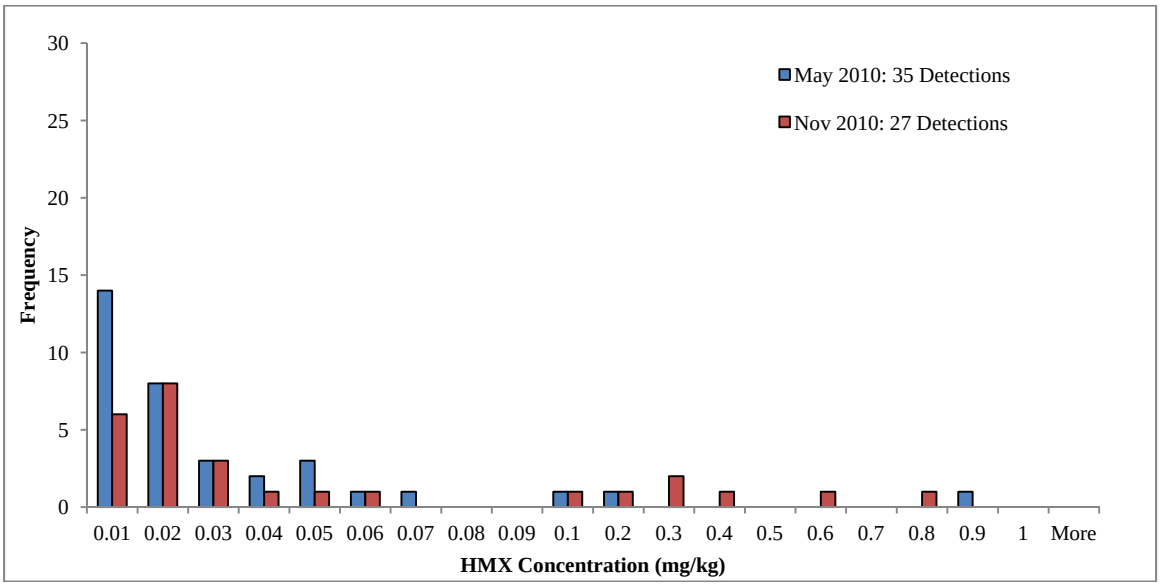


Figure 98. Frequency histogram of HMX soil concentrations found in the unplanted region of Plot #1 with LC/MS only through the May and November 13-14, 2010 samplings.

A general linear model was applied to the data in order to determine if plant type (planted or unplanted), time (sampling date), or a combination of the two had a statistically significant effect on HMX concentrations in the soil of Plot #1. Non-detections were not included because no parametric test can be performed due to the lack of normality in the distribution of the data as seen in Figure 99 and Figure 100. The results, shown in Figure 101, show that the plant type crossed with time is statistically significant (P-value = 0.003). This means that the planted and

unplanted regions of Plot #1 had a statistically significant impact on the change in concentration of HMX in soil over time.

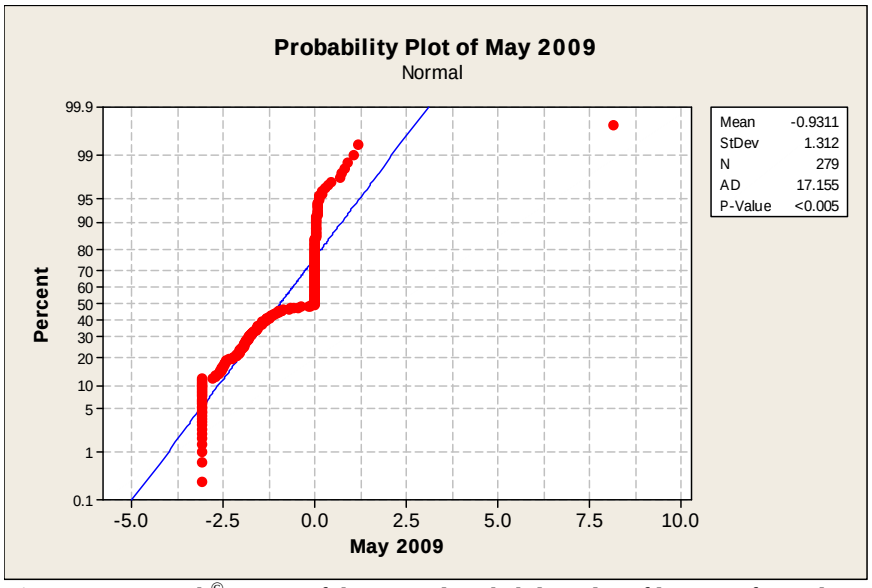


Figure 99. Minitab© output of the normal probability plot of log transformed HMX soil concentrations from the May 24-25, 2010 sampling analyzed using LC/MS.

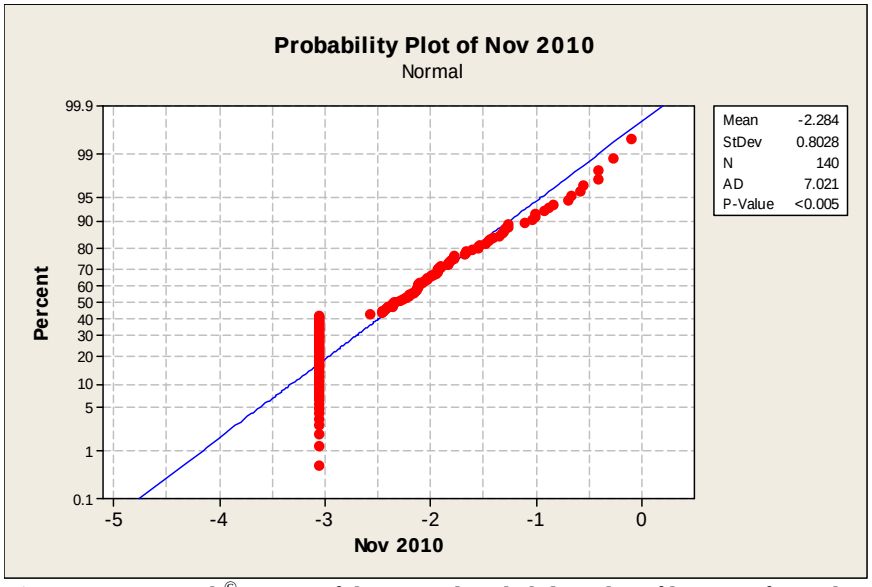


Figure 100. Minitab© output of the normal probability plot of log transformed HMX soil concentrations from the November 13-14, 2010 sampling analyzed using LC/MS.

General Linear Model: Log(HMX Conc) versus Plant Type, Time

```
Factor       Type   Levels  Values
Plant Type   fixed       2  0, 1
Time         fixed       2  1, 2

Analysis of Variance for Log(HMX Conc), using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS   Adj MS      F      P
Plant Type         1   0.0704   0.0000   0.0000   0.00  0.996
Time               1   0.2770   0.0292   0.0292   0.07  0.791
Plant Type*Time    1   3.8667   3.8667   3.8667   9.36  0.003
Error            184  75.9903  75.9903   0.4130
Total            187  80.2044

S = 0.642644   R-Sq = 5.25%   R-Sq(adj) = 3.71%

Unusual Observations for Log(HMX Conc)

      Log(HMX
Obs      Conc)       Fit   SE Fit  Residual  St Resid
  6   0.91177  -1.54199  0.07574   2.45376      3.85 R
 16  -0.16020  -1.54199  0.07574   1.38179      2.17 R
 45   0.07445  -1.54199  0.07574   1.61644      2.53 R
 67  -0.12980  -1.54199  0.07574   1.41219      2.21 R
 72   0.03033  -1.54199  0.07574   1.57232      2.46 R
 98  -0.07259  -1.84930  0.10863   1.77671      2.81 R
136  -0.41844  -1.82306  0.08745   1.40462      2.21 R
181  -0.10255  -1.51474  0.12368   1.41219      2.24 R

R denotes an observation with a large standardized residual.
```

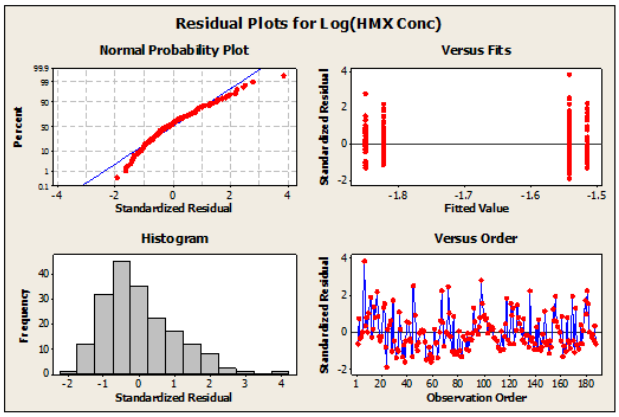


Figure 101. Minitab© output of the application of a General Linear Model on log-transformed HMX soil concentrations analyzed using LC/MS.

The results shown in Figure 86 and Figure 87, Figure 97 and Figure 98, and Figure 101 all indicate that implementation of Bahiagrass Pensacola for phytoremediation is indeed reducing HMX concentrations in soil in the planted region of Plot #1. This conclusion differs from the one reached following the analysis of the data from samples analyzed using HPLC. The conclusions may be different due to the much higher sensitivity of LC/MS to HMX as compared to HPLC.

As shown in Table 13 and Table 15, the limit of detection of HMX using LC/MS and HPLC are 0.002 and 0.04 mg/kg, respectively. The higher sensitivity provides a greater ability to detect trends in the data given the widespread contamination of HMX at low concentrations in Plot #1. However, only two samplings were analyzed using LC/MS. In order to prove that the trend holds true, at least one more sampling should be analyzed. If the Bahiagrass Pensacola is

reducing HMX concentrations in the soil of Plot #1, it is likely that the HMX is translocating to the blades without any transformation. Leaching of HMX from dying or dead plant material has also been observed in published work (Yoon et al., 2002). This is a cause for concern if phytoremediation is to be implemented for the treatment of HMX.

Task 8. Bi-Annual Sampling and Analysis of Vegetation from Field Study Plots

The May 26-27, 2009 sampling was completed before the Bahiagrass Pensacola sod was installed, therefore the first sampling of plants was completed during the November 18-19, 2009 sampling. The detections of explosive compounds found in the discrete plant samples taken from Plot #1 are shown in Figure 102 for the November 18-19, 2009 sampling. HMX and RDX were the only two compounds found in the plants. Of the 100 discrete plant samples, there were 3 detections of HMX ranging in concentration form 0.001 to 0.002 mg/kg and 22 detections of RDX ranging from 0.003 to 0.049 mg/kg.

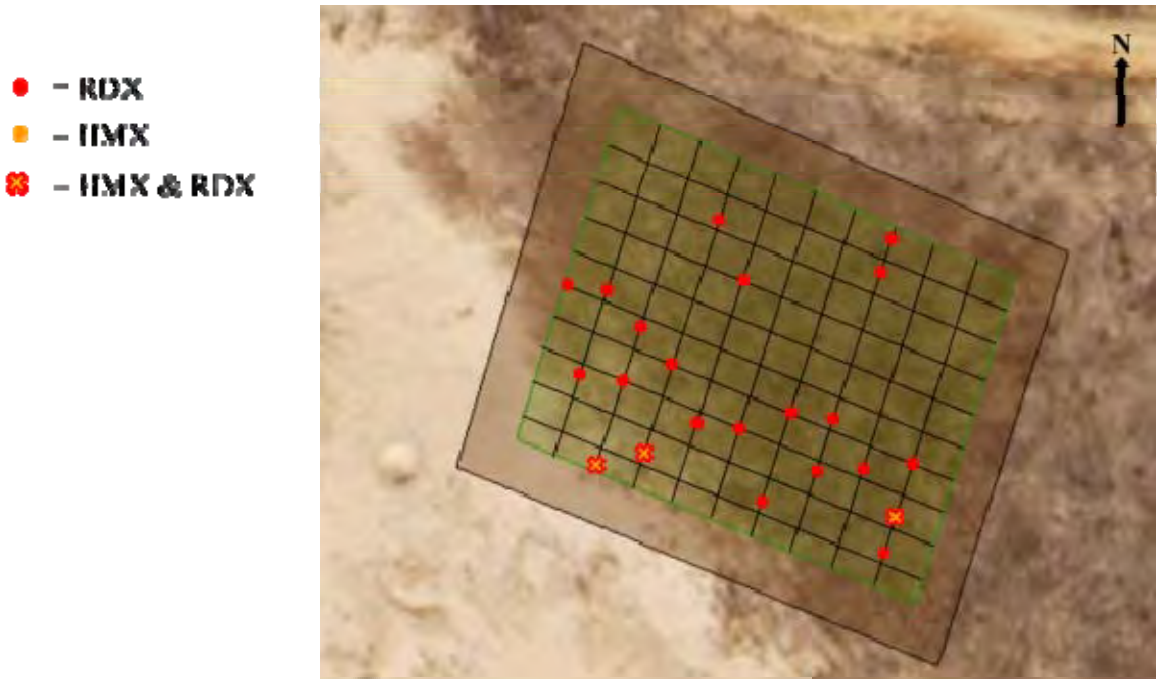


Figure 102. Plot #1 detections in plant tissue for the November 18-19, 2009 sampling analyzed with LC/MS. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

The detections of explosive compounds found in the discrete plant samples taken from Plot #1 are shown in Figure 103 for the May 24-25, 2010 sampling. HMX and RDX were the only two compound found in the plants. Of the 100 discrete plant samples, there were 6 detections of HMX ranging in concentration from 0.033 to 0.670 mg/kg and 5 detections of RDX ranging from 0.014 to 0.122 mg/kg.

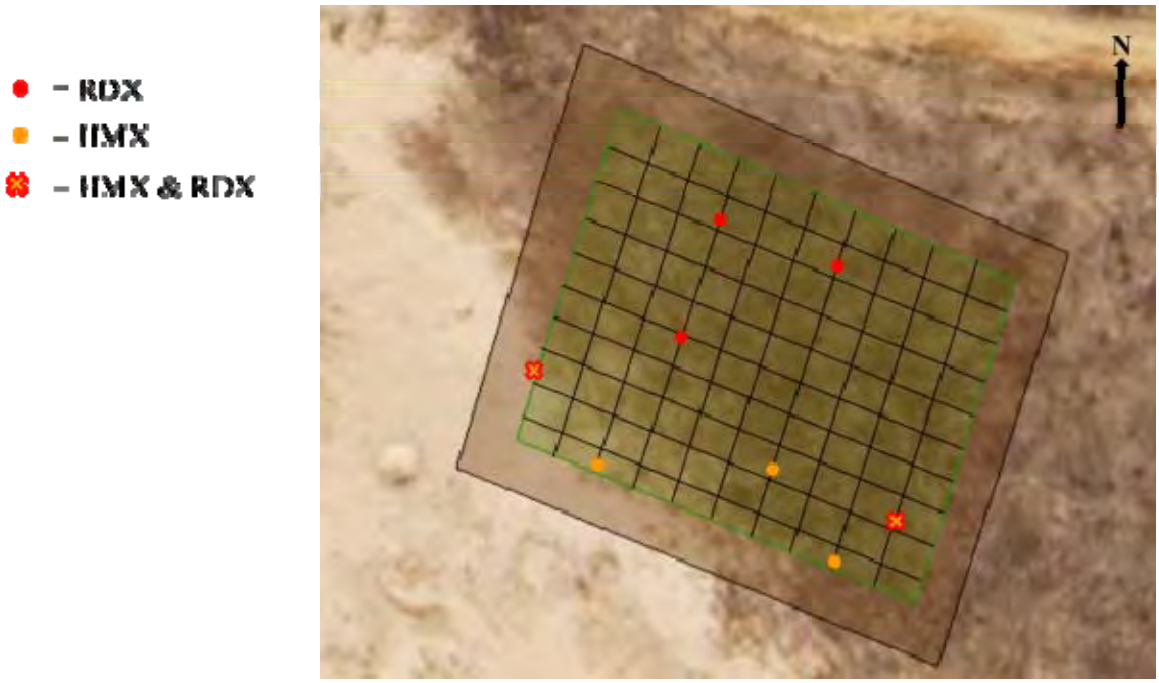


Figure 103. Plot #1 detections in plant tissue for the May 24-25, 2010 sampling analyzed with LC/MS. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

The detection of an explosive compound found in the discrete plant samples taken from Plot #1 is shown in Figure 104 for the November 13-14, 2010 sampling. Of the 100 discrete plant samples, HMX was the only compound detected at a concentration of 0.141 mg/kg.

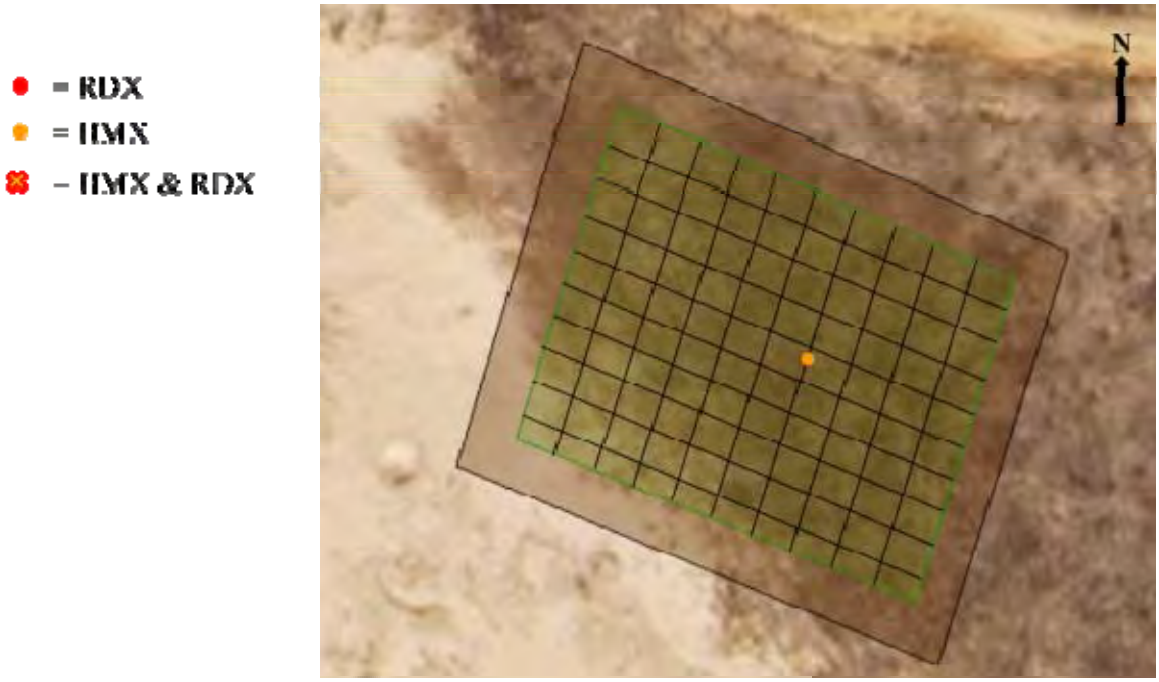


Figure 104. Plot #1 detections in plant tissue for the November 13-14, 2010 sampling analyzed with LC/MS. The region shaded is green is the planted portion. The fringe around the edge is unplanted (12 ft wide).

Figure 102 through Figure 104 show the detections of HMX and RDX in the discrete plant samples taken during the November 18-19, 2009 through November 13-14, 2010 samplings. The general trend is toward fewer detections in the plants of Plot #1. In November 18-19, 2009 there were 22 detections of RDX and 3 detections of HMX. Between the November 18-19, 2009 and May 24-25, 2010, Plot #1 burned down due to detonations of UXO at the adjacent OB/OD site on March 15, 2010 as shown in Figure 63. The vegetation was substantially re-established by the May 24-25, 2010 sampling as shown in Figure 64. However, any explosives accumulated in the grass from the November 18-19, 2009 sampling to March 15, 2010 would not have been observed in the May 24-25, 2010 sampling. The May 24-25, 2010 sampling resulted in 5 detections of RDX and 6 detections of HMX. In November 13-14, 2010, there was only 1 detection of HMX. The results suggest RDX is migrating downward faster than the plants can uptake the contaminant which would support the conclusions drawn from the HPLC data for RDX. There were too few detections of HMX to determine a trend.

Though the detection of explosive compounds in Bahiagrass was significant because it is the first time uptake and translocation of RDX and HMX has been documented during a phytoremediation field study on military ranges, the fraction of total mass found in Bahiagrass as compared to that found in soil is almost insignificant as shown in Table 16. Several assumptions were made in order to determine the mass fractions. One assumption was that the depth of contamination was 5 cm deep – a property of soil contaminated with explosives as described in published material (Hewitt et al., 2007). The amount of plant material present at each sampling was estimated to be 0.1 tons per acre. The mean soil concentration of each contaminant used to calculate the total mass in the soil and grass included non-detects as half the limit of detection.

Table 16. Percent total mass of RDX and HMX in the soil and plant material. Assumptions include a contaminated depth of 5 cm and 0.1 ton per acre of growth. Non-detects were included in means as half the limit of detection.

	$M_{T,RDX}$		$M_{T,HMX}$	
	Soil	Grass	Soil	Grass
November 2009	99.993%	0.007%	99.9996%	0.0004%
May 2010	99.981%	0.019%	99.996%	0.004%
November 2010	99.974%	0.026%	99.998%	0.002%

VI. CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH/IMPLEMENTATION

The overall objective of the research was to understand the mechanisms by which toxic energetic compounds, known to be susceptible to biodegradation, are actually detoxified in contaminated subsurface soils at DoD firing ranges by plants native to the site, either by direct uptake and transformation in plant tissues, or by microbial activity in the rhizosphere. The specific objectives of the research were to determine: (1) whether plants significantly improve biodegradation of explosives using actual soils and plants from representative sites; (2) the respective contribution of plants and soil microbes in the process; and (3) whether the aging of explosives affects the biodegradation process. Additionally, a field-scale implementation of phytoremediation was performed. The specific objectives of this field study are to: (1) determine if the implementation of phytoremediation study significantly improves the biodegradation of explosives in soil through a field study; (2) determine whether plants can significantly uptake and degrade explosives in the field; and (3) compare fate and transport processes in laboratory studies using actual soils from the site of the field study with the field demonstration results.

Phytoremediation of TNT in Soil from Eglin AFB using a Grass Mixture and Poplar Plants

Studies were conducted to assess the phytotoxicity and metabolism of TNT in native plant species *Panicum vigratum* (switchgrass) Alamo and *Paspalum notatum* (Bahiagrass) Pensacola. Phytotoxicity and uptake of TNT by the native grasses was tested using autoradiography and hydroponic studies. A novel method for autoradiography using phosphor imager technology was developed. Both grasses removed TNT from solution efficiently. Uptake and translocation of TNT and RDX was observed using autoradiography.

Native or non-invasive plants, including *Panicum vigratum*, *Paspalum notatum* Pensacola, and *Populus deltoides x nigra,* DN34, were combined with native soils in a microcosm study to assess phytoremediation of TNT in a laboratory scale environment with specific characteristics of Eglin AFB. Characterization of the soil types present at the site of contamination was carried out to assess the availability of these compounds to the plant. One soil was an organic-rich clayey soil and the other was a sandy soil. Soil type and aging play important roles in biodegradation of TNT. The effects of aging were more pronounced in the organic-rich clayey soil, as anticipated. Plants took up TNT from soil systems, but less effectively than in hydroponic systems.

The microcosm study of phytoremediation of TNT at Eglin AFB provides insight into the influence of soil type, aging effects, and planting in a phytoremediation project. Accounting for such factors will aid in determining the potential for successful phytoremediation application at Eglin AFB. The impacts of native soils on phytoremediation systems are made evident in this research. Persisten TNT at Eglin AFB may be accounted for by the lack of microbial activity in Lakeland soil and lack of bioavailability in Dorovan muck. Lakeland soil covers the majority of Eglin AFB with pockets of Dorovan muck found where water movement is stalled and decay or organic living matter is promoted. It may be possible for TNT to be transported as water moves through Lakeland soil and then accumulate in the pockets of Dorovan muck found around the base. Aging of TNT further decreases bioavailability and increases the time it will persist, markedly so in Dorovan muck. TNT that has been again in soils will likely have a different sorption isotherm and therefore it will be more difficult to model its fate in the environment.

The approach used in this research can be extended to selection of other plants or sites with varying soil properties to assist in determining the potential for phytoremediation of TNT. Working within a site's ecosystem is likely to improve the success of phytoremediation for the containment and removal of TNT. Native plant species will be better adapted to the climate and soils in which they are found and as an *in situ* treatment they will not threaten the livelihood of other plant species. Many cases of invasive plant species expose the danger of introducing new plant species into an ecosystem. Research should show that native plants are capable of tolerating and taking up TNT and possible degrading it. The emerging techniques using phosphor imager autoradiography are certainly useful tools for studying uptake of TNT and TNT metabolites in new plant species. It provides a quick assessment tool for uptake of TNT and this work has incorporated a semi-quantitative method for analysis.

The work at Eglin AFB investigates phytoremediation holistically and representatively. This research attempts to account for all of the many factor influencing phytoremediation at Eglin AFB and use factors that are specific to the site. The tools and methods developed in this research lay groundwork for creating standards in phytoremediation research. Sites of interest for phytoremediation should be fully characterized from native soils to native plant species in order to devise a fully integrated approach and predict the success of the treatment technology.

Biodegradation of Explosives in Unplanted Soils from Eglin AFB

Biodegradation experiments performed in this research have addressed the degradation potential for RDX, HMX, and TNT in unplanted soils native to Eglin AFB. Microcosm studies were performed using the two main soil types present at Eglin AFB, Lakeland Soil and Dorovan Muck. Contaminated soil used in the experiments was kept at conditions similar to that of surface soil at Eglin AFB. Soil was incubated at warm, moist, and aerobic conditions throughout the 56 day experiment. RDX and HMX were shown to be recalcitrant under these experimental conditions in both soil types. These results were expected due to similar observations presented in the literature for these compounds under aerobic conditions. TNT degraded relatively quickly to 2-ADNT and 4-ADNT in both soil types which were freshly contaminated. TNT degraded much more slowly in Dorovan Muck which had been contaminated and aged for 18 months, likely due to the high clay content of this soil. These results show that bioremediation or natural attenuation may be a viable treatment strategy for TNT contaminated soil without significant clay content. Conversely, RDX and HMX are recalcitrant under field conditions in Eglin AFB soils and require alternative treatment strategies such as phytoremediation to ensure sustainable range management.

Phytoremediation of RDX in Soil from Eglin AFB using Bahiagrass and Poplar Plants

The laboratory microcosm study showed significant reductions in the concentration of RDX in native Eglin AFB soil in the presence of Bahiagrass Pensacola and hybrid poplar. The concentration of RDX in the presence of Bahiagrass decreased an average of 98.6% after 56 days. The concentration of RDX in the presence of hybrid poplar decreased an average of 99.1% after 40 days. There was no reduction in RDX soil concentrations in the excised root microcosms which suggest plant exudates and decomposing root material did not enhance biodegradation in the soil. There were no RDX detections in the root and blade or leaf tissue samples. This is contradictory to published material on the uptake of RDX that has shown that due to its high solubility and mobility in the environment, RDX is readily translocated to leaves.

In the future, it is suggested that laboratory studies incorporate flow-through in order to better represent conditions at Eglin AFB.

Gene Expression by Hybrid Poplar Exposed to TNT

We have demonstrated several new genes that are implicated in the detoxification and metabolism of TNT by Populus. In particular, we have confirmed the "green liver" model of different gene families being expressed during the time course experiments demonstrating successive Phase I transformation, Phase II conjugation, and Phase III compartmentation processes. Many of the genes identified in this study were related to those significantly expressed in previous Arabidopsis studies, indicating the similarity between these plants. However, genes specifically involved in the uptake and transformation of TNT in hybrid poplar trees were identified here. These genes included the significantly upregulated cupin, glucosyltransferases, and protein transport families. Also in this research we observed several interesting downregulation patterns in the areas of respiration, citric acid cycle, shikimatic pathway, and toxic responses. This is the first report of genes cupin, phosphofructokinases, and glucosyltransferases, being implicated in the detoxification and metabolism of TNT by Populus.

Characterization of the Microbial Community from Soils at Eglin AFB

The microbial communities of the two soil types present at Eglin AFB were determined by Terminal Restriction Fragment Length Polymorphism (T-RFLP). Dorovan Muck contained considerably more microbial diversity than Lakeland Soil, likely due to the higher organic content in Dorovan Muck. Dorovan Muck contained more phylogenetic classifications and relative abundance was more evenly distributed among different taxonomic levels than in Lakeland Soil. Proteobacteria was the dominant phylum type present in both soil types. The low microbial diversity present in Lakeland Soil indicates that bacteria which could be introduced for bioremediation purposes may not be able to compete with the current microbial population.

Phytoremediation Field Study for the Treatment of Explosive Compounds at Eglin AFB

Important findings were made involving the three most prevalent explosive compounds found at military testing and training ranges: TNT, RDX, and HMX. The number of TNT detections was low throughout the entire study. The mean concentrations of TNT and TNT metabolites showed that mean TNT concentrations remained low (at or near the limit of detection) while mean TNT metabolite concentrations increased over time in both the planted and unplanted regions of Plot #1. This was shown in data analyzed by both HPLC and LC/MS. From these results, it was concluded that the microbial communities in the soil are successfully degrading the compound. This result is supported by work completed in the lab, which showed the degradation of TNT and the formation of metabolites in unplanted Eglin AFB soil (Anderson, 2010). It is unclear if the implementation of phytoremediation is enhancing this process because rates could not be determined between the planted and unplanted regions of Plot #1.

The frequency histograms of each sampling, the mean concentrations of contaminants, and the general linear model applied to the data showed the RDX concentrations in soil were decreasing in both the planted and unplanted regions of Plot #1. This suggests the RDX is migrating downward and likely into the groundwater faster than the Bahiagrass is able to uptake and translocate the compound. This is conceivable given the solubility of RDX in water and its mobility in the environment. The results of the field study are contradictory to the results from

the laboratory microcosm study. This is likely due to the watering regime of the laboratory study. Special care was given so that the soil was moist while ensuring only a minimal amount of water drained from the pots. RDX was not given the ability to be transported out of the root zone. This is not the case in the environment where the water will percolate through the soil and provide recharge to groundwater.

There were mixed results for the fate of HMX in the field study. The data from the analysis of samples by HPLC indicated that the HMX was migrating downward with little or no treatment from the Bahiagrass as exhibited by the histograms and general linear model. However, the data from the analysis by LC/MS, which is much more sensitive to HMX, showed that the HMX was indeed treated by the Bahiagrass because the concentrations were increasing in the unplanted region while the concentrations in the planted region were decreasing. This was shown by the histograms of each sampling, mean HMX concentrations of each sampling, and a general linear model. However, only two samplings were analyzed using LC/MS. Additional samplings would need to be analyzed to verify this trend.

HMX and RDX were detected in the discrete plant samples retrieved during each sampling. This is significant because it is the first time uptake and translocation of RDX and HMX had been documented during a phytoremediation field study on military ranges. But, the RDX detections in Bahiagrass decreased as the detections in the soil decreased.

Assuming that the airborne deposition rates of explosive compounds were relatively similar in both the planted and unplanted regions of Plot #1, the data suggests that the organic carbon associated with the Bahiagrass roots and sod were effective in preventing or retarding the downward migration and percolation of contaminants. This alone, even without unequivocal proof of phytoremediation, is very positive.

Overall, the objective of implementing phytoremediation as a strategy for the containment or treatment of explosive contaminants on testing and training ranges at Eglin AFB produced negative results. It does not appear TNT is capable of migrating offsite in significant quantities due to its low mobility and biodegradation. However, both HMX and especially RDX pose a great risk of migrating offsite in significant quantities due to their high mobility. It does not appear that phytoremediation using Bahiagrass Pensacola in Lakeland soil at Eglin AFB can significantly treat or contain HMX or RDX.

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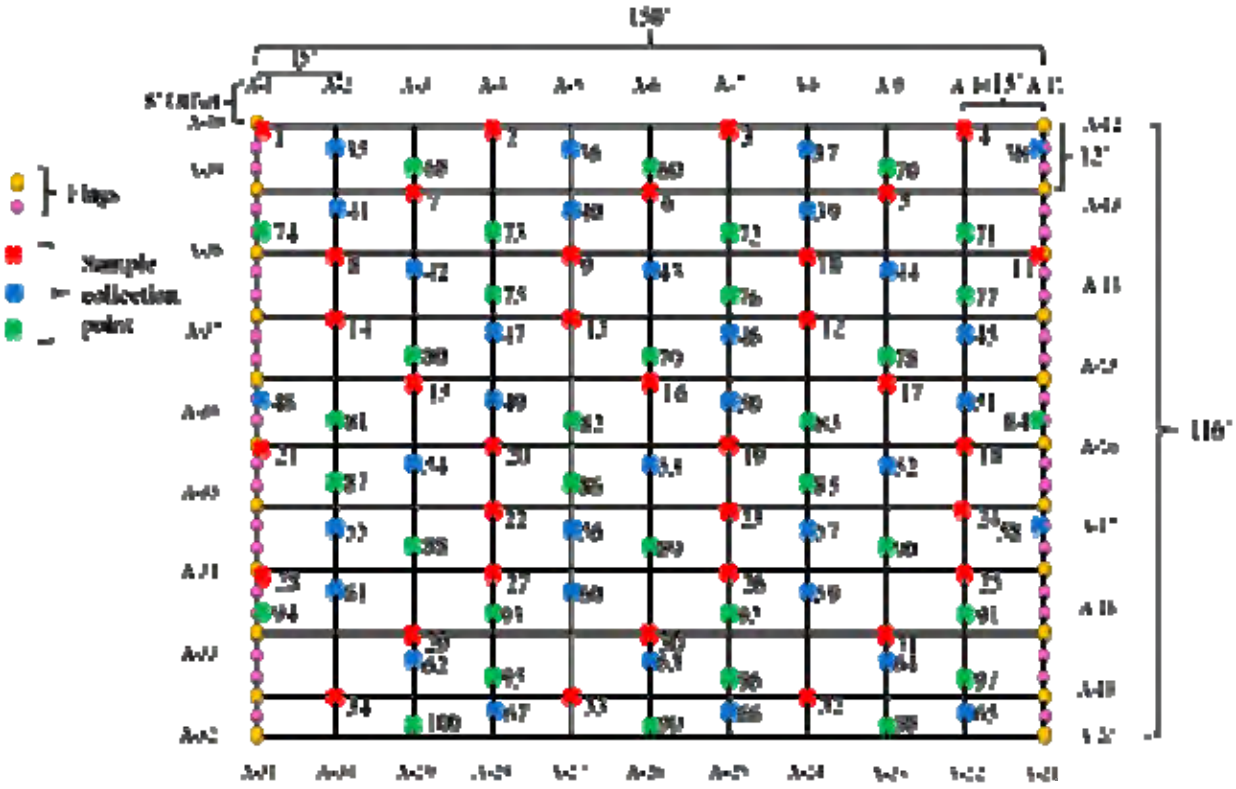
APPENDIX A

SUPPORTING DATA

Reference Figure for Sampling Locations



May 26-27, 2009 HPLC Plot #1 Soil Data

	Soil Concentration (mg/kg)							
Sample	**HMX**	**RDX**	**TNT**	**TNB**	**2-ADNT**	**4-ADNT**	**2,4-DNT**	**2,6-DNT**
1-1	0.20	3.28	0.23					
1-2								
1-3								
1-4								
1-5		0.13						
1-6								
1-7								
1-8								
1-9								
1-10								

1-11								
1-12								
1-13								
1-14	0.05	0.33						
1-15								
1-16		0.15						
1-17								
1-18								
1-19								
1-20								
1-21	0.04	0.56						
1-22	0.22	0.46						
1-23								
1-24								
1-25								
1-26								
1-27								
1-28								
1-29	0.15	0.82						
1-30		0.06						
1-31	0.10							
1-32								
1-33	5.78	0.16						
1-34								
1-35			0.16					
1-36								
1-37								
1-38								
1-39								
1-40								
1-41								
1-42	0.09	0.20						
1-43		0.16						
1-44								
1-45		0.43						
1-46		0.22						
1-47								
1-48								
1-49								

1-50								
1-51								
1-52								
1-53		0.19						
1-54		0.17						
1-55	0.84	3.71						
1-56								
1-57								
1-58								
1-59		0.14						
1-60								
1-61								
1-62								
1-63								
1-64								
1-65		0.14						
1-66	0.07	0.35						
1-67	0.63	0.91						
1-68								
1-69								
1-70		0.14						
1-71								
1-72	0.29	1.15						
1-73	0.88	0.22						
1-74								
1-75								
1-76								
1-77		0.17						
1-78								
1-79								
1-80	0.14	0.08						
1-81								
1-82								
1-83								
1-84								
1-85								
1-86	0.90	0.16						
1-87								
1-88								

1-89								
1-90								
1-91	0.12	0.14			0.24		29.91	1.82
1-92								
1-93		0.20						
1-94	0.53	0.40		0.12				
1-95								
1-96								
1-97								
1-98								
1-99								
1-100	0.81	2.20	0.11	0.33	0.10		5.27	0.49
A-1								
A-2								
A-3								
A-4	0.37	0.27						
A-5								
A-6								
A-7								
A-8								
A-9								
A-10								
A-11								
A-12								
A-13								
A-14	0.05	0.24						
A-15								
A-16								
A-17								
A-18								
A-19								
A-20								
A-21								
A-22								
A-23								
A-24								
A-25		0.31						
A-26	0.05							
A-27	15.79	154.30						

A-28		0.16						
A-29	0.17							
A-30		2.74						
A-31	0.27							
A-32	0.32	0.37						
A-33	0.54	1.45	0.21	0.97			1.09	
A-34	0.23	0.52						
A-35	0.05	0.11						
A-36	0.05	0.12						
A-37								
A-38	0.12	0.11	0.12	0.38	0.19	0.27	15.02	1.00
A-39		0.07						
A-40								

November 18-19, 2009 HPLC Plot #1 Soil Data

	Soil Concentration (mg/kg)							
Sample	**HMX**	**RDX**	**TNT**	**TNB**	**2-ADNT**	**4-ADNT**	**2,4-DNT**	**2,6-DNT**
1-1	0.11							
1-2				0.50				
1-3								
1-4								
1-5								
1-6								
1-7								
1-8								
1-9		0.14						
1-10								
1-11	0.14		0.46					
1-12								
1-13								
1-14								
1-15								
1-16								
1-17								
1-18								
1-19								
1-20								
1-21	0.11	0.20						
1-22		0.18						
1-23								
1-24								
1-25		0.86						
1-26								
1-27								
1-28								
1-29	0.13	0.15						
1-30								
1-31								
1-32								
1-33	0.06	0.09						
1-34								
1-35								

1-36								
1-37								
1-38								
1-39								
1-40								
1-41								
1-42								
1-43								
1-44								
1-45								
1-46								
1-47	0.04	0.11						
1-48		0.08						
1-49								
1-50		0.12						
1-51	0.07	0.20						
1-52		0.11						
1-53		0.15						
1-54	0.23	0.33						
1-55	0.29	1.31						
1-56								
1-57								
1-58								
1-59								
1-60	0.05							
1-61	0.21	0.35		0.08				
1-62	0.06	0.06						
1-63	0.04	0.06						
1-64								
1-65	0.06	0.08						
1-66	0.08	0.16						
1-67	0.30	0.12						
1-68		0.16						
1-69		0.09						
1-70								
1-71								
1-72	0.05	0.05						
1-73	0.06	0.07						
1-74		0.05						

1-75								
1-76								
1-77								
1-78								
1-79								
1-80		0.16	0.09					
1-81	0.04	0.06						
1-82								
1-83		0.06						
1-84								
1-85								
1-86								
1-87			0.05					
1-88								
1-89		0.10						
1-90								
1-91		0.19						
1-92								
1-93								
1-94	3.35	17.13		0.18			0.23	
1-95	0.08	0.15						
1-96								
1-97								
1-98								
1-99								
1-100	0.35	1.04		0.04				
A-1	0.05	0.04						
A-2								
A-3								
A-4								
A-5								
A-6								
A-7		0.04						
A-8								
A-9		0.12						
A-10		0.04						
A-11								
A-12								
A-13								

A-14								
A-15	0.08	1.82		0.05				
A-16								
A-17								
A-18	0.11	0.09						
A-19								
A-20		0.05						
A-21								
A-22								
A-23								
A-24								
A-25	0.42	1.04						
A-26								
A-27	0.07	0.14						
A-28		0.06						
A-29	0.05	0.05						
A-30								
A-31	0.04							
A-32	0.18	0.09						
A-33	0.18	0.15	0.04				1.28	
A-34	0.34	0.73		0.08				
A-35	0.08	4.12						
A-36	0.08	0.13						
A-37	0.07	0.09						
A-38	0.14	0.10						
A-39								
A-40		0.09						

November 18-19, 2009 LC/MS Plot #1 Plant Data

November		
Sample	**HMX (µg/kg)**	**RDX (µg/kg)**
V1-1		
V1-2		
V1-3		
V1-4		
V1-5		
V1-6		
V1-7		
V1-8		
V1-9		
V1-10		
V1-11		
V1-12		
V1-13		8.9
V1-14		
V1-15		
V1-16		
V1-17		
V1-18		
V1-19		
V1-20		
V1-21		25.5
V1-22		42.5
V1-23		39.2
V1-24		35.7
V1-25		
V1-26		29.0
V1-27		33.2
V1-28		
V1-29		
V1-30		
V1-31		
V1-32		
V1-33		
V1-34		
V1-35		
V1-36		
V1-37		3.3
V1-38		
V1-39		48.9
V1-40		
V1-41		
V1-42		
V1-43		
V1-44		
V1-45		
V1-46		
V1-47		
V1-48		
V1-49		
V1-50		
V1-51		
V1-52		
V1-53		
V1-54		8.2
V1-55		
V1-56		
V1-57		
V1-58		
V1-59		5.4
V1-60		7.9
V1-61		14.6
V1-62		
V1-63		
V1-64		
V1-65		
V1-66		
V1-67		
V1-68		
V1-69		
V1-70		
V1-71		
V1-72		
V1-73		47.7
V1-74		

V1-75		
V1-76		
V1-77		
V1-78		
V1-79		
V1-80		
V1-81		5.3
V1-82		
V1-83		
V1-84		
V1-85		5.0
V1-86		
V1-87		
V1-88		
V1-89		
V1-90		8.9
V1-91	1.4	10.0
V1-92		
V1-93		
V1-94		
V1-95	2.2	16.7
V1-96		7.4
V1-97		10.9
V1-98		
V1-99		
V1-100	1.3	11.3

May 24-25, 2010 HPLC Plot #1 Soil Data

	Soil Conc. (mg/kg)						
Sample	HMX	RDX	2,4,6-TNT	4-ADNT	2-ADNT	2,6-DNT	2,4-DNT
1-1		0.17629609					
1-2							
1-3							
1-4		0.13721126					
1-5							
1-6						0.06939871	0.15838958
1-7							
1-8							
1-9							
1-10							
1-11							
1-12							
1-13			0.06454312				
1-14	6.16004745	0.21247302					
1-15							
1-16							
1-17							
1-18							
1-19			0.04639495				
1-20	0.04371278	0.02444794	0.03713433				
1-21							
1-22	0.34416135						
1-23			0.12448058				
1-24							
1-25		0.02440426	0.08179216				
1-26	0.19146235						
1-27	0.03828993	0.03494344					
1-28	0.44911022	2.06413634					
1-29		0.14407383					
1-30							
1-31							
1-32							
1-33	0.11039066	0.25782764	0.06656077				
1-34	0.22158861						
1-35							
1-36							
1-37			0.12672852				

1-38							
1-39							
1-40							
1-41							
1-42	0.12760368	0.29096943					
1-43		0.18571465					
1-44							
1-45							
1-46							
1-47							
1-48							
1-49							
1-50							
1-51							
1-52							
1-53							
1-54	0.02404641						
1-55							
1-56							
1-57							
1-58							
1-59							
1-60							
1-61	0.7274274	0.88461877					
1-62	0.0547813						
1-63							
1-64							
1-65							
1-66							
1-67							
1-68							
1-69							
1-70							
1-71							
1-72	0.20628752						
1-73	0.1100158						
1-74							
1-75							
1-76							
1-77							
1-78							

1-79							
1-80	0.30200764						
1-81							
1-82							
1-83							
1-84							
1-85							
1-86							
1-87							
1-88							
1-89							
1-90							
1-91							
1-92							
1-93							
1-94	0.74893365	1.17736034					1.71084855
1-95	0.07976106						
1-96							
1-97							
1-98							
1-99							
1-100	1.14016588						
A-1							
A-2	0.15278494						
A-3							
A-4							
A-5	0.1565067						
A-6							
A-7							
A-8							
A-9							
A-10							
A-11							
A-12	0.08604354						
A-13							
A-14	0.12379583						
A-15							
A-16							
A-17							
A-18	0.08224203						
A-19							

A-20	0.11816239						
A-21							
A-22							
A-23							
A-24							
A-25							
A-26							
A-27							
A-28	0.07102849						
A-29							
A-30	0.20055142						
A-31	0.15753292						
A-32	0.05461602						
A-33	0.07433761						
A-34	0.05929844						
A-35							
A-36	0.08304206						
A-37							
A-38							
A-39	0.11365053						
A-40	2.96514928						

May 24-25, 2010 LC/MS Plot #1 Soil Data

	Soil Conc. (mg/kg)						
Sample	2-ADNT	4-ADNT	HMX	RDX	2,4-DNT	2,6-DNT	2,4,6-TNT
1-1							
1-2							
1-3							
1-4			0.01058278	0.07833008			
1-5							
1-6			0.0807984	0.11257134			
1-7			0.0159681				
1-8			0.02023303				
1-9			0.02811689				
1-10							
1-11							
1-12							
1-13							
1-14	0.04874911	0.06717936	8.16142004				
1-15			0.04793316				
1-16			0.0910091				
1-17							
1-18							
1-19			0.02860903				
1-20			0.1256247				
1-21							
1-22			0.43994787	0.15362647			
1-23			0.01750932				
1-24							
1-25			0.0362205				
1-26			0.21510099				
1-27			0.09289039				
1-28			0.69150949	0.61256939			
1-29	0.05159587	0.045271	0.10239564	0.07964672			
1-30			0.0194722				
1-31			0.0143771				
1-32			0.02024688				
1-33			0.20081638	0.08192167			
1-34			0.28323418				
1-35			0.00870506				
1-36			0.00167764				
1-37			0.03526003				
1-38	0.00577295	0.00730355	0.04858927				

1-39							
1-40			0.07074409				
1-41	0.02070002	0.02424029	0.00603468				
1-42			0.36249837	0.37670651			
1-43			0.01446781	0.19678707			
1-44			0.00373581				
1-45			0.00736922				
1-46	0.01575068	0.02649849	0.00592442				
1-47			0.13882626	0.31068117			
1-48			0.03697684				
1-49							
1-50			0.00400637				
1-51	0.00722699	0.00994063	0.00984414				
1-52			0.00265742				
1-53	0.00287455	0.00275565	0.01392592	0.06445201			
1-54	0.00531116	0.00816393	0.06545041	0.17966933			
1-55	0.00905343	0.01418657	0.06131429	0.14429927			
1-56			0.01601911				
1-57			0.01306019				
1-58			0.0037958				
1-59							
1-60							
1-61	0.02070884	0.04103276	1.18699791	1.8730425			
1-62	0.00741327	0.01096216	0.10605824	0.09628008			
1-63			0.00917535				
1-64			0.00649				
1-65			0.01228268	0.05100043			
1-66			0.02533724	0.02989769			
1-67			0.03310837	0.05356402			
1-68							
1-69							
1-70			0.02758682				
1-71							
1-72			0.02933655	0.15250288			
1-73			0.01312619				
1-74			0.00295611				
1-75							
1-76							
1-77			0.00334533				
1-78							
1-79							

1-80							
1-81			0.00878381				
1-82							
1-83			0.00475924				
1-84			0.00263515				
1-85			0.00362235				
1-86							
1-87	0.03217159	0.04475606	0.0361536	0.08676956			
1-88			0.01199592	0.05347675			
1-89			0.01200912	0.08283609			
1-90			0.00370207				
1-91			0.02658293	0.06453561			
1-92			0.06956538				
1-93							
1-94	0.02060807	0.03447344	0.74165108	1.34763952		1.821037	
1-95	0.00938379	0.01358084	0.05804982	0.09561845			
1-96			0.00898086				
1-97	0.00357799	0.00342612	0.02804726	0.05183604			
1-98							
1-99			0.02556558				
1-100	0.01096701	0.01411553	1.07232336	0.07828143			
A-1	0.00725277	0.00959957	0.06248949	0.32822635			
A-2			0.00943212				
A-3			0.00292676				
A-4			0.04815535				
A-5			0.0023945				
A-6			0.00288611				
A-7			0.00232616				
A-8							
A-9							
A-10			0.01539706				
A-11			0.0033374				
A-12			0.00196155				
A-13			0.00195786				
A-14			0.00969882				
A-15			0.00733785				
A-16							
A-17			0.00340385				
A-18			0.01222019				
A-19			0.00328192				
A-20			0.00838404				

A-21			0.00739786				
A-22			0.01303005				
A-23							
A-24			0.09520392				
A-25	0.00837492	0.00957061	0.0304255	0.06681174			
A-26	0.00333855	0.00573739	0.01599481	0.04393074			
A-27			0.01105791	0.02510751			
A-28			0.02053854	0.02703894			
A-29	0.01205055	0.01538285	0.04508854	0.05976683			
A-30	0.01123517	0.01525407	0.8460737	0.0763097			
A-31							
A-32	0.00559141	0.00766177	0.13394553	0.26626268			
A-33	0.00779554	0.01575115	0.05180351	0.10651542			
A-34	0.00265007	0.00382821	0.03772362	0.07299448			
A-35	0.00394197	0.00562325	0.04214314				
A-36			0.01230395	0.06259448			
A-37			0.02534416	0.07425982			
A-38			0.01736312				
A-39			0.02086873				
A-40			0.01816653				

May 24-25, 2010 LC/MS Plot #1 Plant Data

	Soil Conc. (mg/kg)	
Sample	HMX	RDX
1-V1		
1-V2		
1-V3		
1-V4		
1-V5		
1-V6		
1-V7		
1-V8		
1-V9		
1-V10		
1-V11		
1-V12		
1-V13		
1-V14		
1-V15		
1-V16		
1-V17		
1-V18		
1-V19		
1-V20		
1-V21		0.05090743
1-V22		
1-V23		
1-V24		
1-V25		
1-V26		
1-V27		
1-V28		
1-V29		
1-V30		
1-V31		
1-V32		
1-V33		
1-V34		
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1-V43		
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1-V46		
1-V47		
1-V48		
1-V49		
1-V50		
1-V51		
1-V52		
1-V53		
1-V54		
1-V55		
1-V56		
1-V57		
1-V58		
1-V59		
1-V60		
1-V61	0.12479966	
1-V62		
1-V63		
1-V64		
1-V65		
1-V66		
1-V67		
1-V68		
1-V69		
1-V70		
1-V71		
1-V72		0.04352627
1-V73		0.12195836
1-V74		
1-V75		
1-V76		
1-V77		

1-V78		
1-V79		
1-V80		
1-V81		
1-V82		
1-V83		
1-V84		
1-V85		
1-V86		
1-V87		
1-V88		
1-V89		
1-V90		
1-V91	0.03335841	0.01353338
1-V92	0.67013608	
1-V93		
1-V94	0.43492324	0.10332401
1-V95		
1-V96		
1-V97		
1-V98	0.21707856	
1-V99		
1-V100	0.13338381	

November 13-14, 2010 HPLC Plot #1 Soil Data

	Soil Conc. (mg/kg)						
Sample	HMX	RDX	2,4,6-TNT	4-ADNT	2-ADNT	2,6-DNT	2,4-DNT
1-1							
1-2							
1-3							
1-4							
1-5							
1-6							
1-7							
1-8							
1-9							
1-10							
1-11							
1-12							
1-13							
1-14							
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1-30							
1-31							
1-32							
1-33							
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1-35							
1-36							
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1-38							
1-39							
1-40							
1-41							
1-42							
1-43							
1-44							
1-45							
1-46							
1-47							
1-48							
1-49							
1-50							
1-51							
1-52							
1-53							
1-54							
1-55	0.25077549	0.40621443					
1-56							
1-57							
1-58							
1-59							
1-60							
1-61							
1-62							
1-63							
1-64							
1-65		0.49856066					
1-66	0.04500215						
1-67							
1-68							
1-69							
1-70							
1-71							
1-72							
1-73							
1-74							
1-75							
1-76							
1-77							
1-78							

1-79							
1-80							
1-81							
1-82							
1-83							
1-84							
1-85							
1-86							
1-87							
1-88							
1-89							
1-90							
1-91					5.13235108		2.50920526
1-92	0.3744554						
1-93							
1-94	0.44922724	0.10670943					
1-95							
1-96							
1-97							
1-98							
1-99							
1-100					0.80424784		
A-1							
A-2							
A-3							
A-4							
A-5							
A-6							
A-7							
A-8							
A-9							
A-10							
A-11							
A-12							
A-13							
A-14							
A-15							
A-16							
A-17							
A-18	0.44153832						
A-19							

A-20							
A-21							
A-22	0.41979859				10.5037721		4.70388318
A-23							
A-24							
A-25							
A-26							
A-27							
A-28	0.35419437						
A-29							
A-30							
A-31	0.24207341						
A-32	0.36349652						
A-33	0.69427692						
A-34	0.34662539						
A-35	0.22834212				1.54769534		0.73219332
A-36							
A-37							
A-38							
A-39		0.8358699					
A-40							

November 13-14, 2010 LC/MS Plot #1 Soil Data

	Soil Conc. (mg/kg)						
Sample	2-ADNT	4-ADNT	HMX	RDX	2,4-DNT	2,6-DNT	2,4,6-TNT
1-1			0.00922176				
1-2							
1-3							
1-4			0.0087828				
1-5			0.00751327				
1-6							
1-7							
1-8							
1-9			0.00459919				
1-10							
1-11							
1-12							
1-13			0.0039854				
1-14			0.00344709				
1-15							
1-16			0.01532132				
1-17							
1-18			0.00367838				
1-19			0.01033946				
1-20							
1-21			0.02894417				
1-22	0.09969832	0.02275823	0.21485555	0.10257021			
1-23							
1-24							
1-25			0.00771584				
1-26			0.00579333				
1-27			0.14640725				
1-28	0.03212919	0.00599116	0.05378867		0.3135009	1.9900557	
1-29	0.03067669	0.00616447	0.0973421				
1-30			0.00550025				
1-31							
1-32			0.01679443				
1-33			0.13228073				
1-34			0.04706328	0.20354169		0.58365453	
1-35							
1-36							
1-37			0.0278549				
1-38							

1-39							
1-40			0.01655937				
1-41							
1-42							
1-43			0.00788725				
1-44							
1-45			0.04479054				0.05050336
1-46							
1-47			0.00629996				
1-48			0.01150429				
1-49							
1-50							
1-51							
1-52							
1-53			0.011688				
1-54			0.00440954				
1-55			0.38155315	0.75414162			
1-56			0.00705705				
1-57							
1-58							
1-59			0.00942048				
1-60			0.00388313				
1-61	0.04844994	0.01481982	0.00586844				0.09501175
1-62			0.02155746				
1-63			0.00358524				
1-64							
1-65							
1-66			0.03953046				
1-67			0.00342477				
1-68							
1-69							
1-70			0.00445418				
1-71							
1-72							
1-73							
1-74							
1-75							
1-76			0.01247283				
1-77							
1-78							
1-79							

1-80							
1-81			0.00681217				
1-82			0.07743989				
1-83							
1-84			0.00513342				
1-85							
1-86			0.00753453				
1-87			0.00271882				
1-88							
1-89			0.00738388				
1-90			0.00439428				
1-91			0.09277721		0.3631482	2.40469067	
1-92							
1-93			0.03619417				
1-94		0.0079689	0.26378908				
1-95			0.03345918				
1-96			0.02144549				
1-97			0.01485875				
1-98							
1-99			0.00744813				
1-100	0.01932134	0.00907176	0.0504514				
A-1			0.0043335				
A-2			0.0098923				
A-3	0.01144886	0.00455678	0.00665462				
A-4							
A-5							
A-6			0.09622586				
A-7							
A-8							
A-9							
A-10			0.01565644				
A-11							
A-12							
A-13							
A-14			0.0123955				
A-15							
A-16							
A-17			0.0082997				
A-18			0.53174571				
A-19							
A-20			0.01458744				

A-21			0.00620789				
A-22			0.20221623		0.91069659	4.65433718	
A-23							
A-24			0.01062195				
A-25			0.00772806				
A-26			0.01167538				
A-27			0.05346584				
A-28			0.01186227				
A-29							
A-30		0.02264494	0.03568038				
A-31		0.01572808	0.38002015				
A-32		0.02796585	0.11959722				
A-33		0.0246052	0.78968365				
A-34	0.05500115	0.0081444	0.27382009				
A-35		0.01309495	0.02854894				
A-36			0.02067583				
A-37			0.02505956				
A-38	0.02336422	0.00233818	0.01661524				
A-39			0.04938164				
A-40			0.01135698				

November 13-14, 2010 LC/MS Plot #1 Plant Data

	Soil Conc. (mg/kg)	
Sample	HMX	RDX
1-V1		
1-V2		
1-V3		
1-V4		
1-V5		
1-V6		
1-V7		
1-V8		
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1-V31		
1-V32		
1-V33		
1-V34		
1-V35		
1-V36		
1-V37		
1-V38		
1-V39		
1-V40	0.1408992	
1-V41		
1-V42		
1-V43		
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1-V93		
1-V94		
1-V95		
1-V96		
1-V97		
1-V98		
1-V99		
1-V100		

APPENDIX B

LIST OF SCIENTIFIC/TECHNICAL PUBLICATIONS

Presentations

Brentner, Laura B. Investigating molecular responses in poplar phytoremediation of TNT: glutathione transferases. (poster) 15th Annual Conference Biopharmaceuticals and Industrial Biotechnology: From Gene Expression to Bioprocessing. October 23-24, 2006. Center for Biocatalysis and Bioprocessing, The University of Iowa, Iowa City, IA.

Tanaka, Sachiyo. Microarray analysis of Arabidopsis in response to RDX treatment: a screening technique to identify RDX up-regulated genes in Populus. (featured speaker) 15th Annual Conference Biopharmaceuticals and Industrial Biotechnology: From Gene Expression to Bioprocessing. October 23-24, 2006. Center for Biocatalysis and Bioprocessing, The University of Iowa, Iowa City, IA.

Publications

Anderson, Travis J. (2010). Phytoremediation of energectic compound at Eglin Air Force Base. Thesis (MS), University of Iowa.

Brentner, Laura B. (2008). Gene Expression of Transferase Enzymes and Environmental Factors Involved in Phytoremediation of 2,4,6-Trinitirotoluene (TNT). Thesis (PhD), University of Iowa.

Brentner, L. B., Mukherji, S. T., Merchie, K. M., Yoon, J. M., Schnoor, J. L., & Van Aken, B. (2008). Expression of glutathione S-transferases in poplar trees (Populus trichocarpa) exposed to 2,4,6-trinitrotoluene (TNT). *Chemosphere, 73*(5), 657-662.

Brentner, Laura B., Mukherji, Sachiyo T., Walsh, Susan A., & Schnoor, Jerald L. (2010). Localization of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) and 2,4,6-Trinitrotoluene (TNT) in Poplar and Switchgrass Plants using Phosphor Imager Autoradiography. *Environmental Pollution,* 158(2), 470-475.

Flannigan, Matthew B. (2011). Phytoremediation for the Treatment of Energetic Material Releases on Testing and Training Ranges at Eglin Air Force Base. Thesis (MS), University of Iowa.

Flokstra, B. R., Van Aken, B., & Schnoor, J. L. (2008). Microtox (R) toxicity test: Detoxification of TNT and RDX contaminated solutions by poplar tissue cultures. *Chemosphere, 71*(10), 1970-1976.

Schnoor, Jerald L. & Krahe, Catherine. (2012). Plants and Endophytes. In L. Newman (Eds.) *Plant-Microbe Interactions for Environmental Remediation* (1st ed.). Hanover: Springer.

Tanaka, Sachiyo. (2007). Gene expression and metabolism of RDX by poplar and the symbiotic role of Methylobacterium populi BJ001. Thesis (PhD), University of Iowa.

Tanaka, S., Brentner, L.B., Merchie, K.M., Yoon, J-M., Schnoor, J.L., and Van Aken, B. (2007). Analysis of Gene Expression in Poplar Trees (*Populus deltoides x nigra*, DN34) Exposed to the Toxic Explosive Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). *International Journal of Phytoremediation*, *9*(1-3), 15-30.

APPENDIX C

OTHER SUPPORTING MATERIALS

Awards

SERDP Project-of-the-Year 2009 (Environmental Restoration)